ROLE OF POLYPEPTIDE pVIII IN BOVINE ADENOVIRUS (BAdV)-3 LIFECYCLE

A Thesis
Submitted to the Faculty of Graduate Studies and Research
in Partial Fulfillment of the Requirements for
the Degree of Doctor of Philosophy
in the
Department of Veterinary Microbiology
University of Saskatchewan
Saskatoon

By
Amit Gaba

PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a postgraduate degree from the University of Saskatchewan, I agree that the libraries of this university may make it freely available for inspection. I further agree that permission for copying of this thesis in any manner, whole or in part, for scholarly purposes may be granted by the professors who supervised my thesis work or in their absence, the Head of the Department or the Dean of the college in which my thesis work was done. It is understood that any copying or publication or use of this thesis or parts thereof for financial gain shall not be allowed without any written permission. It is also understood that due recognition shall be given to me and to the University of Saskatchewan in any scholarly use which may be made of any material in my thesis.

Request for permission to copy or to make other use of material in this thesis in whole or part should be addressed to:

Head of the Department of Veterinary Microbiology

University of Saskatchewan,

Saskatoon, Saskatchewan,

S7N 5B4

ABSTRACT

Bovine adenovirus-3 (BAdV-3) a member of genus *Mastadenovirus* was first isolated in England from the eye of seemingly healthy cattle (Darbyshire *et al*., 1965). The linear double stranded genome of BAdV-3 is of 34446 bp in length and is organized into early, intermediate and late regions (Reddy *et al*., 1998). The mRNAs produced from late region are grouped into seven families (L1-L7) based upon usage of polyadenylation sites (Reddy *et al*., 1998).The L6 region of BAdV-3 encodes pVIII protein a structural protein associated with hexon connecting the core with the adenovirus capsid (Reddy *et al*., 1998,Russell., 2009). The objective of the present study was to characterize interaction of pVIII with cellular protein eukaryotic initiation factor 6 (eIF6) and to determine the role of pVIII in the life cycle of BAdV-3.

Our initial yeast two-hybrid results indicated that pVIII interacts with cellular protein eukaryotic initiation factor 6 (eIF6). We validated our yeast two-hybrid results using GST pull-down assay, co-immunoprecipitation assay and bimolecular fluorescence complementation (BiFC) assay. Moreover, BAdV-3 late protein pVIII interacts with eIF6 in transfected and BAdV-3 infected cells. Analysis of the interaction of mutant BAdV-3 pVIIIs with eIF6 using co-immunoprecipitation assay identified amino acids 147 to 174 of BAdV-3 pVIII involved in interaction with eIF6. Similar analysis of plasmids expressing mutant eIF6 proteins identified amino acids 44 to 97 of eIF6 involved in interaction with BAdV-3pVIII.

The eukaryotic initiation factor 6 is a 245 amino acid protein that is important both for ribosome biogenesis and protein translation (Miluzio *et al*., 2009). It specifically binds to free 60S ribosomal subunit and prevents its joining with 40S ribosomal subunit (Gartmann *et al*., 2010,Russell & Spremulli., 1979,Valenzuela *et al*., 1982). Here, we report a novel interaction between BAdV-3 pVIII and cellular protein eIF6, which appears to alter the formation of

functional 80S ribosomes and may modulate the cellular protein translation. The polysome profile analysis of uninfected and BAdV-3 infected MDBK cells indicated that there are more free 60S subunits in BAdV-3 infected cells as compared to uninfected cells suggesting the inhibition in joining of 40S and 60S ribosomal subunits. Comparison of polysome profiles of VIDO GT1 (stable cell line expressing BAdV-3 pVIII) and VIDO DT1 (not expressing BAdV-3 pVIII) cells also revealed the presence of more free 60S and free 40S subunits and reduction in functional 80S ribosomes in pVIII expressing cells compared to VIDO DT1 cells. These results suggest that the presence of pVIII impairs joining of 40S and 60S subunits, which may lead to reduction in formation of functional 80S subunits.

To study the biological significance of pVIII-eIF6 interaction, we constructed a recombinant BAdV-3-d147-174, expressing mutant pVIII (containing deletion of domain interacting with eIF6). Analysis of the growth kinetics of BAdV-3-d147-174 suggested that growth of mutant virus was significantly affected. Moreover, $CsCl_2$ gradient purification and TEM analysis revealed the significant decrease in the formation of mature virus particles in BAdV-3-d147-174 infected cells. Analysis of viral gene expression revealed that while the expression of early gene product (DBP) was not affected, the expression of all the analyzed late adenovirus proteins was significantly decreased in the cells infected with BAdV-3-d147-174. However, there was no significant difference in the incorporation of analysed structural proteins between BAdV-304a and BAdV-3-d147-174. These results indicate that abrogating the interaction of pVIII with eIF6 affects expression of adenovirus late proteins, which may result in decreased production of progeny virus. We speculate that interaction of pVIII and eIF6 plays a major role in preferential translation of adenoviral mRNAs during the late phase of infection.

Proteolytic maturation involving cleavage of six structural and one non-structural precursor protein is an important aspect of adenovirus life cycle. However, it is not known what role each individual cleavage event plays in determining adenovirus particle stability and infectivity. Analysis of amino acid sequence of BAdV-3 pVIII identified two potential adenovirus protease cleavage sites. Our results suggest that BAdV-3 pVIII is cleaved by adenovirus protease at both potential consensus cleavage sites. Further, while abrogation of cleavage at only one site does not affect the formation of mature virus, the absence of cleavage at both sites appears lethal for the production of progeny virus. Moreover, the cleavage of pVIII at both potential cleavage sites is a major contributing factor in producing stable BAdV-3 particles. Together, these studies provide evidence that proteolytic maturation of pVIII is essential for correct virus assembly and stability of the adenovirus particle.

ACKNOWLEDGEMENTS

On the outset, I would like to thank my advisor Dr. Suresh K. Tikoo for accepting me in the program and providing me the opportunity to work in his laboratory. His invaluable and critical suggestions, scholarly guidance, active persuasion, supervision and encouragement had been the constant source of inspiration throughout the course of my study and research work. I also wish to thank the members of my advisory committee Dr. Yan Zhou, Dr. Alexander Zakhartchouk, Dr. Linda Chelico and graduate chair Dr. Janet Hill for their interest, unreserved guidance and valuable inputs and suggestions.

I would especially like to thank Dr. Niraj Makadiya, Satyender Hansra, Dr. Robert Brownlie, Wayne Connor and Dr. Patricia Gonzalez Cano for their help and suggestions throughout the project. I am thankful to Veterinary Microbiology staff, VIDO technical and administrative staff for helping me whenever I needed their help. I would also like to thank past and present members of Dr. Tikoo's laboratory and fellow graduate students especially Niraj, Lisanework, Siddharth, Ravindera, Azhar, Pankaj, Sanjeev, Xin, Tekele, Jeeva, Kyle, Abdo, Vani, Sugandhika, Mingji, Kuan, Elisa, Sharmin, Akanksha, Maria, Shermila and other members of the Vectored Vaccine Laboratory for their support, suggestions, friendship and providing a positive environment.

I would also like to thank my dearest parents, my in laws, my sisters and my brothers in law for their kind blessings, love, patience, overwhelming support and encouragement. I would also like to thank my wife Supriya for being so patient and understanding, for her unconditional love, support and encouragement. I would also like to thank my daughter Ananya for being my stress buster and for giving me all the happiness and joy. Last but not the least I would like to

thank Almighty for giving me the patience and strength to remain stoic during the course of my study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS USED IN THIS THESIS

4E-BP	eIF4E Binding Protein
ADP	Adenovirus Death Protein
AIDS	Acquired Immunodeficiency Syndrome
AP	Alkaline Phosphatase
BAdV	Bovine Adenovirus
BiFC	Bimolecular Fluorescent Complementation
BoMtch1	Bovine Mitochondrial carrier homolog 1
BoPSAP	Bovine Presenilin-1-Associated Protein
bp	Base pair
CAR	Coxsackie and adenovirus receptor
CBP	CREB-binding protein
CD	Cluster of Differentiation
CPE	Cytopathic Effect
CTL	Cytotoxic T Lymphocytes
DAPI	4'-6-Diamidino-2-phenylindole
DBP	DNA Binding Protein
DDX	DEAD Box Protein
DEAD	Aspargine-Glutamine-Alanine-Aspargine
DMEM	Dulbeco's Minimum Essential Medium
DYNLT1	Dynein light chain Tctex-type 1
E	Early
E2F	E2 Promoter Factor
EFL1	Elongation Factor Like 1
eIF	Eukaryotic Initiation Factor
eIF4G	Eukaryotic Initiation Factor 4G
eIF6	Eukaryotic Initiation Factor 6
EMCV	Encephalomyocarditis Virus
ER	Endoplasmic Reticulum

FBS	Fetal Bovine Serum
GFP	Green fluorescent protein
GST	Glutathione-S-transferase
HAdV	Human adenovirus
HIV	Human immunodeficiency virus
IPTG	Isopropyl-β-D-thiogalactopyranoside
IRES	Internal Ribosome Entry Site
ITR	Inverted Terminal Repeat
kb	Kilo base
kDa	Kilodalton
MAb	Monoclonal antibody
MDBK	Madin-Darby Bovine Kidney
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MLP	Major late promoter
MLTU	Major late transcriptional unit
Mnk1	MAP kinase-interacting serine/threonine-protein kinase 1
MMP	Mitochondrial membrane potential
MOI	Multiplicity of infection
mTOR	Mammalian target of rapamycin
NFBP	NFkB-binding protein
NLS	Nuclear localization signal
NPC	Nuclear Pore Complex
ORF	Open Reading Frame
PABP	Poly A Binding Protein
PAdV	Porcine Adenovirus
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PC	Preinition Complex
PCR	Polymerase chain reaction
PI3K	Phosphoinositide-3-OH kinase

PKR	Protein Kinase R
PML	Promyelocytic Leukemia
Pol	Polymerase
pTP	Preterminal protein
RACK1	Receptor for Activated Kinase C
Rb	Retinoblastoma
RGD	arginine-glycine-aspartic acid
RID	Receptor Internalization and degradation
SDS	Sodium dodecyl sulfate
SV40	Simian Virus 40
$TCID_{50}$	Tissue culture infectious $dose_{50}$
Tctex-1	T-complex-associated testis expressed -1
TNF	Tumor necrosis factor
TPL	Tripartite leader
UTR	Untranslated Region
VA RNA	Virus-associated RNA
VSV	Vesicular stomatitis virus
WSAdV	White sturgeon adenovirus

1.0 LITERATURE REVIEW

1.1 Adenoviruses

Adenoviruses are large, nonenveloped viruses that have linear double stranded DNA (26 to 45kb) as their genome (Davison *et al*., 2003) enclosed in an icosahedral capsid (Berk., 2013). Adenoviruses were first isolated in early 1950's by two distinct groups that were trying to find the etiologic agents of acute respiratory infections (Hilleman & Werner., 1954,Rowe *et al*., 1953). Although adenoviruses have been isolated from a wide variety of species including mammals, birds, reptiles and fishes, their replication is mostly limited to their natural host (Shenk., 2001). The in-depth molecular characterization of adenovirus infected cells have contributed significantly in understanding various biological processes like replication of DNA, gene expression (both cellular and viral), cell cycle, splicing mechanism and cellular growth regulation (Berk., 2013).

1.1.1 Adenovirus classification

Adenoviruses are members of family *Adenoviridae* which is further grouped into five genera: *Mastadenovirus, Aviadenovirus, Ataadenovirus, Siadenovirus* and *Ichtadenovirus.* The genus *Mastadenovirus* consists of 25 different species of adenovirus that can infect mammals only (Brown *et al*., 2012). Traditionally serology, phylogenetic analyses and more recently genome organization has been used to distinguish members of this genus from other genera of *Adenoviridae* family. Distinguishing features of *Mastadenoviruses* include considerably longer (93-371 bp) and more complex ITRs (Brown *et al*., 2012) and presence of unique genes that code for genus specific proteins like core protein V, cementing protein IX and some of the proteins encoded by E1A, E1B, E3 and E4 regions (Davison *et al*., 2003,Vellinga *et al*., 2005). *Human*

adenovirus C is the type species for genus *Mastadenovirus.* The biological properties like replication and organization of genome have been studied in great details for some of the isolates of *Human adenovirus C* species (Brown *et al*., 2012).

The genus *Aviadenovirus* consists of 8 species of adenoviruses that can only infect birds. Distinguishing features of aviadenoviruses include considerably larger genomes that range between 43804 bp (FAdV-1) (Chiocca *et al*., 1996) and 45667 (FAdV-4) (Griffin & Nagy., 2011). The aviadenovirus virion contain two fibers per vertex inspite of the fact that some of them have two genes for fiber protein while others have only one gene that encode for fiber protein (Brown *et al*., 2012). Unlike *Mastadenoviruses,* aviadenoviruses lack the E1 and E3 region, and genes coding for proteins V and IX (Brown *et al*., 2012). Compared to other genera the E4 region of aviadenoviruses is long (Davison *et al*., 2003). Though a number of transcription units that are exclusive to aviadenoviruses are present on the right end of aviadenovirus genome, most of the genes and their products encoded by this region still remain uncharacterized. Members of genus *Aviadenovirus* are pathogenic and are associated with diseases in birds like inclusion body hepatitis, bronchitis and hydropericardium syndrome. *Fowl adenovirus A* is the type species for genus *Aviadenovirus* (Brown *et al*., 2012).

The genus *Atadenovirus* consists of 5 species of adenovirus (Brown *et al*., 2012). Members of genus *Atadenovirus* have a broad host range and can infect birds, ruminants, marsupial and reptiles (Davison *et al*., 2003). Distinguishing features of *Atadenoviruses* include high A+ T content of the genome, comparatively shorter genome than *Mastadenoviruses* and absence of proteins V and IX (Benkö & Harrach., 1998). All members of the genus *Atadenovirus* have unique structural proteins called p32K (ÉlŐ *et al*., 2003) and LH3 (Gorman *et al*., 2005,Pantelic

et al., 2008). The LH3 forms knob like structures on surface of virion (Pantelic *et al*., 2008). *Ovine adenovirus D* is the type species for genus *Atadenovirus* (Brown *et al*., 2012).

The genus *Siadenovirus* consists of 5 species of adenoviruses. Distinguishing features of *Siadenoviruses* include short genomes and short ITRs (Kovács & Benkő., 2009,Pitcovski *et al*., 1998). *Siadenoviruses* are so named because of their unique feature of encoding sialidase enzyme (Davison *et al*., 2003). Unlike *Mastadenoviruses, Siadenovirus* genome lacks the E1, E3 and E4 regions and the genes encoding for proteins V and IX. *Frog adenovirus* is the type species for genus *Siadenovirus* (Brown *et al*., 2012).

The genus *Ichtadenovirus* consists of only one species of adenovirus known as white sturgeon adenovirus 1 (WSAdV-1). The genome of WSAdV-1 is 48395 bp long which makes it the longest genome among all known adenoviruses (Brown *et al*., 2012).

1.1.2 Human adenovirus

Human adenovirus (HAdV) was first isolated from human adenoids in the 1950s (Rowe *et al*., 1953). The extensive characterization of HAdVs for over 6 decades have led to the identification of 67 HAdV types (Ghebremedhin., 2014), which are further subdivided into 7 subgroups (A to G) based on the property of virus to agglutinate the erythrocyte of different animals, oncogenecity in animals, transformation of cultured cells, genomic sequencing and bioinformatics analysis (Berk., 2013,Ghebremedhin., 2014).

1.1.2.1 Virion structure

Adenoviruses are large, non-enveloped double stranded DNA viruses that have a typical icosahedral capsid that surrounds a DNA containing core (Fig.1.1.1). The icosahedral AdV capsid which has a diameter of approximately 90nm is composed of 13 virion proteins (Berk., 2013,San Martín., 2012). Hexon protein with 720 copies per virion is the major building block of

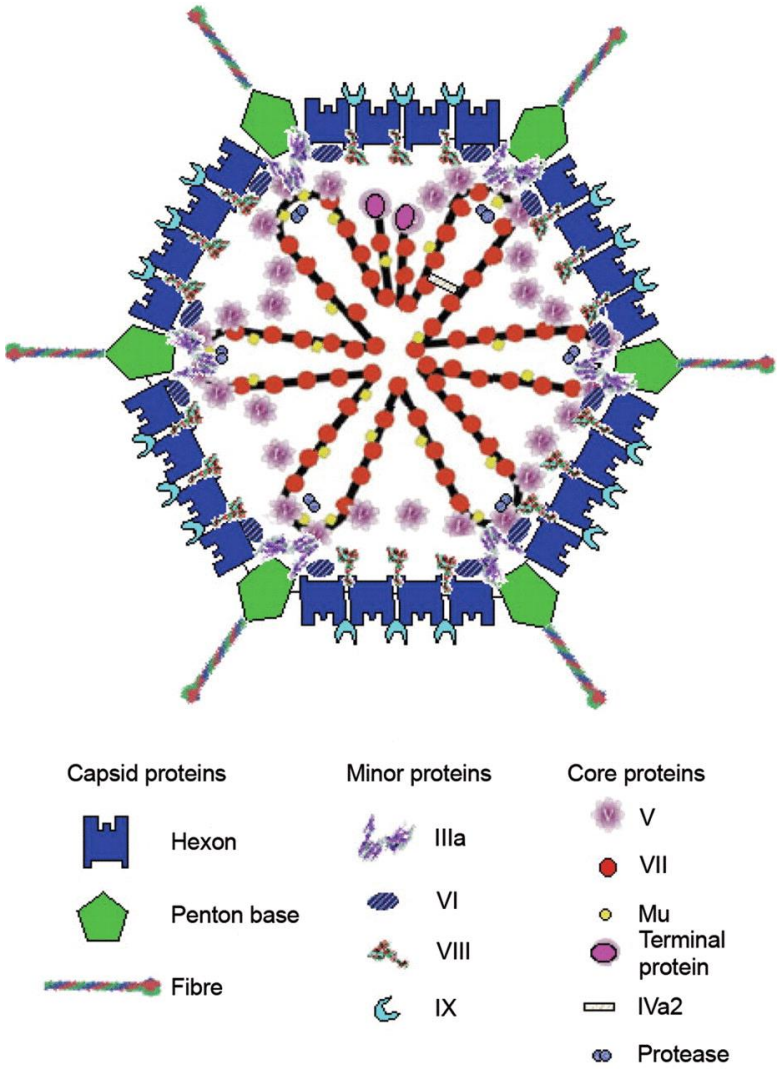


Figure 1.1.1 Schematic representation of adenovirus structure. Reprinted from (Russell., 2009) with permission from publisher (Microbiology Society).

the capsid. Each capsid has 240 trimers of hexon protein with 12 trimers present at each capsid facet. A penton capsomere composed of 5 copies of penton protein is present at each of the 12 vertices making a complex with a trimer of protruding fiber protein (Russell., 2009,Vellinga *et al*., 2005). Along with these three major capsid proteins, four minor capsid proteins namely pIIIa, pVI, pVIII and IX are also present in the virion (Fig.1.1.1). The polypeptide IIIa has a copy number of 60 per virion. It is present at the inner surface of the capsid and interacts with hexon, protein pVI and protein pVIII (Liu *et al*., 2010,San Martín., 2012). The polypeptide VI is an important protein present at the inner surface of capsid with a copy number of 360 per virion. It interacts with proteins IIIA, hexon and V (Moyer *et al*., 2015,San Martín., 2012,Vellinga *et al*., 2005). Polypeptide VIII is another minor protein that is present at the inner surface of capsid. It has a copy number of 120 per virion and stabilizes the virion structure by providing a link between peripentonal hexons and the rest of the capsid (San Martín., 2012,Vellinga *et al*., 2005). Protein IX with a copy number of 240 is the only minor protein exposed on the outside of the capsid (Vellinga *et al*., 2005). Interactions of these minor proteins with other minor and major capsid proteins stabilize the virion structure (Berk., 2013).

The adenovirus core is composed of viral DNA genome and core proteins that include protein VII, V, Mu (X), IVa2, terminal protein (TP) and adenovirus protease (Fig.1.1.1). Polypeptide VII with more than 800 copies per virion is the most abundant core protein (van Oostrum & Burnett., 1985). It is highly basic and rich in arginine. Protein V and Mu are the other basic and arginine rich components of the core which along with polypeptide VII bind to viral DNA and condense it (Chatterjee *et al*., 1986,Hosokawa & Sung., 1976). Protein V binds to genomic DNA and polypeptide VI and thus acts as a bridge between capsid and core (Matthews & Russell., 1998a,Reddy & Nemerow., 2014). Core protein IVa2 is present at only one vertex of

the icosahedron and is involved in the encapsidation of viral DNA (Christensen *et al*., 2008,Ostapchuk *et al*., 2005,Tyler *et al*., 2007). The terminal protein present as only two copies per virion binds covalently to the 5′ ends of the viral DNA (Rekosh *et al*., 1977). The adenovirus protease functions to cleave selected viral precursor proteins during virion maturation (Greber., 1998).

1.1.2.2 Virus life cycle

Since viruses lack their own metabolic machinery, they depend entirely on living host cell to carry out their life-cycle. To take control of the host cell metabolic machinery, they need to enter the cell. Initial contact between host cell and a virus involves attachment of the viral surface components to the cell surface receptor(s). These initial interactions in many cases are non specific and electrostatic, primarily aimed at providing the virus with an opportunity to recruit specific receptors that can provide virus entry into the cell (Grove & Marsh., 2011).

For adenoviruses, the knob portion of fiber is the surface component responsible for establishing the initial contact with the host cell. For HAdV species A, C, D, E and F fiber knob interacts with coxsackie and adenovirus receptor (CAR) (Fig.1.1.2) (Arnberg., 2012,Bergelson *et al*., 1997). CAR, a protein that belongs to immunoglobulin family is a trans-membrane protein that contains an extracellular domain, a trans-membrane domain and an intracellular domain (Coyne & Bergelson., 2005). However, unlike other HAdV's, species B HAdV can use CD80, CD86 or CD46 as their receptor (Marttila *et al*., 2005,Short *et al*., 2006). Moreover, sialic acid has also been reported as receptor for some species D adenoviruses (Arnberg *et al*., 2000) In addition to these well-known receptors, other host cell factors such as heparin sulfate proteoglycans, lactoferrin and coagulation factors have been reported to bind to adenovirus and could serve as functional adenovirus receptors (Arnberg., 2009).

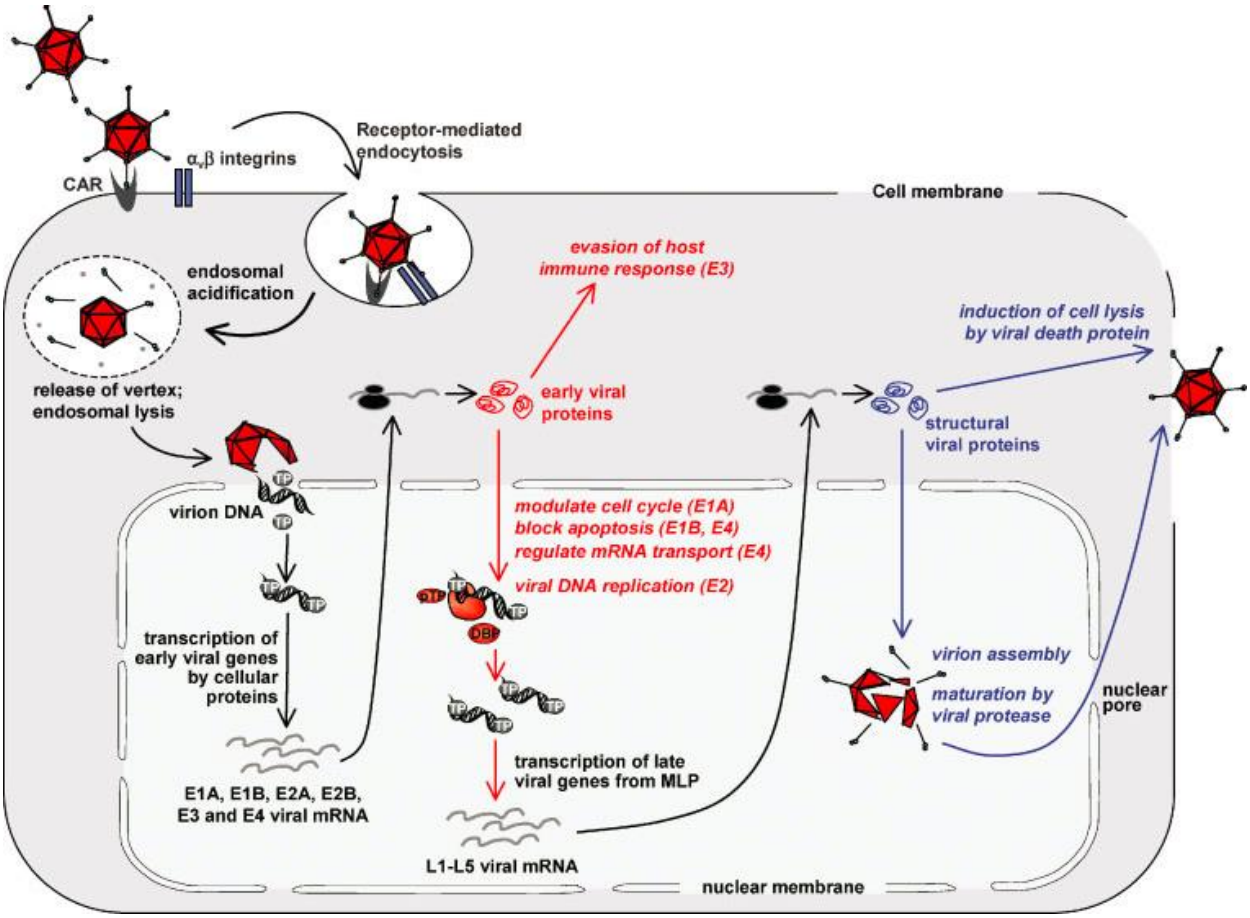


Figure 1.1.2 Schematic representation of adenovirus infection pathway. Reprinted from (Lenaerts *et al*., 2008) with permission from John Wiley and Sons (publisher).

The interaction of CAR and fiber is mainly involved in attachment of virus to cell receptor and has no role in the internalization of adenovirus particles into host cell (Wickham *et al*., 1996). For efficient internalization of adenovirus particles into host cell, the interaction involving the arginine-glycine-aspartic acid (RGD) motif of penton and integrins present on the cell surface is required (Bai *et al*., 1993,Wickham *et al*., 1993). This interaction activates the phosphoinositide-3-OH kinase, p130CAS and Rho GTPases, which are involved in rearrangement of cytoskeleton required for the endocytosis of the virion. This endocytic process is mediated by clathrin and requires GTPase, dynamin and the adaptor protein 2 (Li *et al*., 1998,Meier & Greber., 2004). After endocytosis, the clathrin coated vesicle mature into the endosomes and the pH of endosome becomes more acidic. The increased acidification of the endosome triggers dismantling of the capsid, causing the release of penton, hexon and the internal capsid proteins IIIa, VI and VIII (Fig. 1.1.2) (Gastaldelli *et al*., 2008,Greber *et al*., 1993). This partial uncoating of the capsid exposes the lytic domain of protein VI which causes disruption of the endosomal membrane and subsequent release of partially uncoated virions into the cytoplasm (Wiethoff *et al*., 2005). On reaching the cytoplasm, the partially disassembled capsid makes use of microtubule motor protein dynein to reach to the nuclear pore complex (NPC). The interaction of hexon with dynein allows the movement of capsid towards the nucleus (Bremner *et al*., 2009,Leopold *et al*., 2000). The capsid docks at NPC by binding to CAN/Nup214 (Trotman *et al*., 2001). For further disassembly at the NPC, virus uses molecular motor kinensin 1(Strunze *et al*., 2011). After the disassembly at NPC, the viral genome in complex with protein VII transports to the nucleus using cellular transport factors such as importin, transportin and histone H1(Hindley *et al*., 2007,Trotman *et al*., 2001,Wodrich *et al*., 2006).

The transcription of early (E) region of adenovirus genome starts once viral genome locates in the nucleus. E1A is the earliest region of viral genome to undergo transcription during early phase of infection and encodes two mRNA's with coterminal 5′ and 3′ ends. The two major E1A protein products termed 289R and 243R have 289 and 243 amino acids, respectively (Avvakumov *et al*., 2004). Two major functions of E1A products are to induce the infected cell to enter S phase, making an environment suitable for the replication of virus and to stimulate the transcription of other early viral genes (Berk., 2013).

E1A is an efficient trans-activator and can stimulate transcription from a number of cellular and viral promoters by associating to the DNA binding domains of transcription factors (Liu & Green., 1994). During early phase of infection, EIA stabilizes p53 by binding to Rb and CBP/p300. Deregulation of cell cycle by E1A and stabilization of p53 stimulates apoptosis (Gallimore & Turnell., 2001).

The E1B transcriptional region encodes two proteins, E1B-19K and E1B-55K, which counteract the proapoptotic effects of E1A (Berk., 2005,Debbas & White., 1993).

The E2 early region encodes three proteins namely, DNA binding protein (DBP), DNA polymerase (Pol) and pre-terminal protein (pTP) that are essential for viral DNA replication .The 59kDa DBP is encoded by E2A region and plays a vital role in DNA replication. During initiation phase, it stimulates the binding of viral DNA polymerase to viral DNA. During elongation phase, it unwinds the viral dsDNA and removes the secondary structures thus increasing the efficiency of Pol (Dekker *et al*., 1997,Lindenbaum *et al*., 1986,van Breukelen *et al*., 2003). The 80kDa pTP encoded by E2B region binds to the 5′ end of each strand of viral dsDNA and functions as a primer to initiate the viral DNA replication (Mysiak *et al*., 2004).

After forming heterodimer with Pol, it translocates to the nucleus and starts viral DNA replication (Fredman & Engler., 1993).

The E3 region of adenovirus encodes several proteins that are not essential for viral replication but are required for subverting the host immune response. The transcription of E3 region is regulated by promoter responsive to E1A (Horwitz., 2004).

The E4 region encodes six proteins that perform a variety of functions during the course of viral infection including the cell cycle modulation, DNA repair, late viral protein synthesis, RNA processing and counteracting antiviral innate immune response (Greer *et al*., 2011,O'Shea *et al*., 2005,Ullman & Hearing., 2008,Weitzman & Ornelles., 2005).

Viral DNA replication begins when E2 gene products have accumulated in sufficient amounts. The inverted terminal repeats (ITRs) present on each end of viral genome have origins of DNA replication where a pre-initiation complex (PIC) composed of viral proteins pTP, AdPol and DBP, and cellular proteins NF1 and Oct1 is formed (Bosher *et al*., 1990,Chen *et al*., 1990,Mul *et al*., 1990). The DNA replication starts after assembly of PIC using pTP as a protein primer. First Pol catalyzes the formation of an ester bond between α- phosphoryl group of deoxycytidine monophosphate (dCMP) and β-OH of a serine in pTP. The 3′-OH group of the newly formed pTP-CMP complex functions as a primer for the production of new strand by Pol. Viral proteins Pol and DBP, and the cellular protein topoisomerase (also known as NFII) is essential for DNA elongation (Challberg *et al*., 1980,Lichy *et al*., 1981,Nagata *et al*., 1983). The production of new viral DNA occurs in 5′ to 3′ direction using strand displacement mechanism (Lechner & Kelly., 1977).

Viral proteins IVa2 and IX are known as delayed early or intermediate proteins since their expression coincides with DNA replication and their transcripts are produced earlier than late

proteins. It has been proposed that during initial stages of infection, the cellular transcriptional repressors suppress the expression of IVa2 and IX. The cellular protein RBP-2N suppresses the expression of protein IX while protein IVa2-RF represses expression of IVa2. As viral DNA replication proceeds, the viral genome copy number increases and hence more binding sites for cellular repressors become available. Eventually the number of binding sites outnumber the cellular repressors, thus relieving the inhibition of protein IX and IVa2 expression (Chen *et al*., 1994,Dou *et al*., 1994,Iftode & Flint., 2004,Lin & Flint., 2000). Protein IVa2 is involved in regulating the activity of major late promoter (MLP) and also plays a role in virus assembly (Lutz & Kedinger., 1996,Zhang & Imperiale., 2003). Protein IX provides the stability to the capsid and is also suggested to play a role in activation of MLP (Lutz *et al*., 1997,Vellinga *et al*., 2005).

After the onset of viral DNA replication, adenovirus late genes start to be expressed efficiently. All late genes are produced from a single transcription unit known as major late transcription unit (MLTU) which is processessed by splicing and usage of differential poly A sites to generate 20 species of late mRNAs (Nevins & Darnell., 1978,Ziff & Evans., 1978). Based on the usage of poly (A) sites these 20 species of late mRNAs are further grouped into five families (L1-L5) (Nevins & Darnell., 1978,Ziff & Fraser., 1978). All late mRNAs share a common 5′ noncoding region known as tripartite leader that helps to increase the translation of the late mRNAs (Berget *et al*., 1977,Chow *et al*., 1977). Major late promoter (MLP) regulates the expression of viral late mRNAs. During early phase of infection, the MLP activity is at very low level but it increases several folds at late times post infection because of the stimulation by viral proteins including IVa2 (Pardo-Mateos & Young., 2004,Tribouley *et al*., 1994) and L4-22K (Backström *et al*., 2010,Morris *et al*., 2010). The products of the late mRNA are mainly viral

structural proteins and scaffolding proteins, which regulate the late phase translation and assembly of virus particle (Berk., 2013).

Assembly of the adenovirus particle occurs in the nucleus but the process begins in cytoplasm itself where assembly of the hexon trimers and penton capsomeres take place. The trimerization of hexon is assisted by L4-100K protein which acts as a scaffold (Hong *et al*., 2005,Horwitz *et al*., 1969,Velicer & Ginsberg., 1970). The assembled hexon and penton capsomeres then moves to the nucleus for final assembly process. In the nucleus, the hexon and the penton capsomeres associate with each other as well as with other minor capsid proteins to form empty capsids (Wodrich *et al*., 2003). Adenovirus DNA is then packaged in these preformed capsids starting with the left end of genome (Hammarskjöld & Winberg., 1980,Hearing *et al*., 1987). The left end of adenovirus genome has seven AT-rich repeat sequences that are reported to be necessary for the encapsidation of viral DNA (Ostapchuk & Hearing., 2003). Adenoviral proteins IVa2, L1 52/55K and L4-22K have been reported to interact with packaging sequence and play a vital role in the encapsidation of viral DNA. Activated adenovirus protease cleaves the selected precursor proteins thus making the progeny virion infectious (Gupta *et al*., 2004,Mangel *et al*., 2003,Weber., 1976).

Although the exact mechanism for the release of adenovirus particle from infected cell is not known, several mechanisms have been postulated that help in the release of adenovirus from infected cells. First, the cellular cytokeratin is cleaved by activated adenovirus protease during the late phase of infection and this destroys the cytoskeleton network of the cell and aids in the release of virus from infected host cells (Brown & Mangel., 2004). Secondly, E3 11.6kDa protein also known as adenovirus death protein (ADP) has been suggested to play a vital role in the lysis of infected cells. While in early phase of infection, ADP is expressed in low quantities

from E3 promoter, at late phase of infection it is expressed at high levels by major late promoter. The accumulation of high amount of ADP in the infected cells subsequently cause death and lysis of the cell (Tollefson *et al*., 1992,Tollefson *et al*., 1996).

1.1.2.3 Human adenovirus pathogenesis

Human adenoviruses can cause a variety of infections and most of them are endemic in nature. Many of these infections are mild or asymptomatic and result in the development of immunity that protect against reinfection with same adenovirus serotype (Walls *et al*., 2003). HAdV's are commonly associated with infection of respiratory system, gastroenteritis and eye infection. They are also associated with urinary tract infection and occasionally can also infect other organs such as liver, pancreas, heart and central nervous system (Wold & Ison., 2013).

Adenoviruses primarily belonging to group C (HAdV serotypes 1, 2, 5 and 6) and occasionally group B (HAdV serotypes 3 and 7) are most commonly associated with upper respiratory tract infection especially in children less than 5 years of age. Approximately 7% of the cases of pediatric upper respiratory tract infection are because of adenovirus infection (Wesley *et al*., 1993,Wold & Ison., 2013). HAdV primarily belonging to group B (serotypes 3 and 7) and frequently group C (serotypes 1, 2 and 5) cause pediatric lower respiratory tract infections leading to pneumonia and bronchiolitis (Hong *et al*., 2001,Kim *et al*., 2003,Pavia., 2011).

Adenoviruses belonging to group B (serotypes 3, 7, 16 and 21) and group E (serotype 4) are reported to cause acute lower respiratory tract infection in adults (Wold & Ison., 2013). HAdV-4 and HAdV-7 are the primary etiological agents for acute respiratory infection in military recruits (Gray *et al*., 2007,Potter *et al*., 2012).

HAdV serotypes 3, 7 and 4 can cause mild conjunctivitis. The infection is usually self-limiting and leads to full recovery (Wadell., 2000). On the other hand, infection of the eye by adenoviruses belonging to group D (serotype 8, 9 and 17) can cause severe epidemic kerato-conjunctivitis (Aoki & Tagawa., 2002,Echavarria., 2009).

Enteric adenoviruses HAdV-40 and 41 have been linked with acute gastroenteritis in children (Wilhelmi *et al*., 2003). HAdV-11 and 21 have been reported to be the cause of acute haemorrhagic cystitis in boys (Mufson & Belshe., 1976,Numazaki *et al*., 1973). Several reports have implicated adenovirus as a cause for myocarditis in children and adults (Bowles *et al*., 2003,Dennert *et al*., 2008,Lozinski *et al*., 1994). In addition to this adenoviruses have been associated with obesity (HAdV-36) (Atkinson *et al*., 2005), intussusception (Bines *et al*., 2006), and meningoencephalitis (Chou *et al*., 1973,Dubberke *et al*., 2006,Kelsey., 1978).

Adenoviruses can frequently infect immune-compromised hosts particularly patients that had undergone organ transplant (Bridges *et al*., 1998,Cames *et al*., 1992,Michaels *et al*., 1992) and patients with acquired immunodeficiency syndrome (AIDS) (Horwitz *et al*., 1984,Krilov *et al*., 1990). Compared to immune-competent host, outcome of adenovirus infection in immune-compromised host can be much more severe and life threatening (Baldwin *et al*., 2000,Leen & Rooney., 2005).

1.1.3 Proten-protein interactions in adenovirus lifecycle

Since viruses do not have their own metabolic machinery, they depend upon host cellular machinery for carrying out various functions. As soon as a virus infects a cell, it takes over the cellular metabolic machinery by means of various interactions with host factors. Interaction of virus and host determines the virus host range and outcome of viral infection. It is therefore

imperative to identify and characterize various virus-host cell interactions to understand the basic biology of a virus (Arnberg., 2009,Wang & Li., 2012,Zhou *et al*., 2009).

Similarly, adenovirus proteins interact with various host proteins and with other adenovirus proteins throughout the viral life cycle to make cellular environment suitable for adenovirus multiplication and to counter host defense mechanism. Study of these adenovirus-host cell interactions resulted in a better understanding of adenovirus biology.

1.1.3.1 Protein-protein interactions during adenovirus attachment and internalization

Adenovirus infectious cycle starts with interaction of fiber knob of adenovirus with the cell surface receptor(s). After attachment of fiber to cell surface receptor, the penton base interacts with integrins, which leads to endocytosis of virion (Arnberg., 2009). Interaction of hexon with microtubule motor protein dynein is essential for transport of partially uncoated virus to nuclear pore complex. At nuclear pore complex (NPC) interaction of hexon with nucleoporin Nup214 is essential for nuclear import of viral DNA (Cassany *et al*., 2015,Trotman *et al*., 2001). The interaction of adenovirus protein IX with kinesin 1 light chain at NPC leads to further disassembly of the virus (Strunze *et al*., 2011). After disassembly at NPC, interaction of pVII with cellular transport factors such as importin and transportin and histone H1 regulates transport of viral genome into the nucleus (Hindley *et al*., 2007,Trotman *et al*., 2001,Wodrich *et al*., 2006)

1.1.3.2 Protein-protein interaction of adenovirus early proteins

The first region of the adenovirus genome to undergo transcription is E1A, which encodes two major proteins namely 289R and 243R. The E1A bind to cellular proteins that regulate E2F family of transcription factors namely retinoblastoma (RB) protein and RB related proteins p130 and 107. The RB and RB related protein represses E2F transcriptional activation by binding to E2F. However, binding of E1A to RB and related proteins displaces them from

E2F and hence RB mediated repression of E2F is relieved allowing the cells to enter S phase of cell cycle (Berk., 2005). E1A has also been reported to interact with several cellular proteins namely p300/CBP, TRRAP/GCN5 and pCAF that are involved in chromatin structure control. These interactions of EIA may increase expression of genes regulating the cell cycle by increasing DNA and transcription factor binding (Chakravarti *et al*., 1999,Hamamori *et al*., 1999,Lang & Hearing., 2003). Moreover, E1A also binds the CDK inhibitors (CKI) p27 and p21. Binding of EIA to CKI's prevent the CKI mediated inhibition of CDK –cyclin complexes thus promoting cell cycling (Chattopadhyay *et al*., 2001,Mal *et al*., 1996,Nomura *et al*., 1998). During early phase of infection, E1A stabilizes p53 partly because of its binding to Rb and CBP/p300. Deregulation of cell cycle by E1A and stabilization of p53 stimulates apoptosis (Gallimore & Turnell., 2001)

The E1B transcriptional region encoded proteins E1B-19K and E1B-55K interact with host \viral proteins to counteract the pro-apoptotic effects of E1A proteins. E1B-55k binds to the p53 activation domain leading to the stearic hindrance for co-activators to interact with p53. Moreover, E1B-55K also has a repressor domain and its binding to p53 converts p53 from gene activator to gene repressor (Martin & Berk., 1998,Yew *et al*., 1994). E1B-55K also causes degradation of p53. It forms a complex with viral protein E4orf6 and cellular proteins Elogin B and C, Culin5 and RBX1. This complex ubiquitin ligase poly-ubiquinates p53 and thus causes its proteosomal degradation (Harada *et al*., 2002,Querido *et al*., 2001). E1B-19K, a homologue of cellular BCL-2 family of proteins mimics anti apoptotic protein MCL1 and, interacts with pro-apoptotic proteins BAK and BAX. Binding of E1B-19K to BAK and BAX prevents their oligomerization and subsequent pore formation, releasing the apoptosis causing factors from the mitochondria (White., 2001).

The E2 early region encoded proteins DNA binding protein (DBP), DNA polymerase (Pol) and pre-terminal protein (pTP) interact with each other to regulate viral DNA replication (as described in section 1.1.2.2) (Mysiak *et al*., 2004,van Breukelen *et al*., 2003).

The E3 region of adenovirus encodes several proteins that are not essential for viral replication but are required for subverting the host immune response. E3-gp19k, a 19kDa protein functions to diminish CTL mediated elimination of adenovirus infected cells. It performs this function by two different mechanisms. Firstly, it binds to MHC class I molecule and prevents its transport to the cell surface. The gp19k protein contains an ER localization signal that prevents transport of MHC class I-gp19k complex to the cell surface (Burgert *et al*., 1987,Horwitz., 2004). Secondly, gp19k binds to TAP and prevents the interaction of TAP and Tapasin. Interaction of TAP and Tapasin is required for effective presentation of antigen by MHC class I molecule. Thus, because of interaction of gp19k and TAP, the cell surface antigen presentation is decreased (Bennett *et al*., 1999,Horwitz., 2004). Moreover, gp19k has been reported to inhibit NK cell activation because of its ability to diminish the cell surface expression of NK cell activating ligands MHC I chain-related protein A and B (McSharry *et al*., 2008). The E3-10.4k and 14.5k proteins also known as RIDα and RIDβ, respectively together form a complex which is called receptor internalization and degradation (RID) complex. The RID complex inhibits apoptosis induced by TNF, Fas ligands and TRAIL by removing their receptors from the cell surface and increasing their internalization and destruction in lysosome (Chin & Horwitz., 2006,Shisler *et al*., 1997,Tollefson *et al*., 1998).

The E4 region encoded proteins perform a variety of functions during the course of viral infection including cell cycle modulation, DNA repair, late viral protein synthesis, RNA processing and counteracting antiviral innate immune response (Greer *et al*., 2011,O'Shea *et al*.,

2005,Ullman & Hearing., 2008,Weitzman & Ornelles., 2005). The E4orf1 protein binds with PDZ domain of many cellular proteins and activates PI3K leading to the activation of mammalian target of rapamycin (mTOR) (Chung *et al*., 2007,Frese *et al*., 2003,O'Shea *et al*., 2005). The E4 orf3 facilitates production and accumulation of late viral mRNA by transporting components of P-bodies to aggresomes (Greer *et al*., 2011). The E4orf4 binds to protein phosphatase 2A and activates mTOR independently of PI3K (O'Shea *et al*., 2005). The E4orf6, a 34kDa protein forms a multifunctional complex with adenovirus E1b55K that is involved in viral DNA replication, processing of RNA, nucleo-cytoplasmic shuttling of late viral mRNA and inhibiting host protein synthesis during viral infection. It also plays a vital role in polyubiquitination and degradation of cellular targets by recruiting cellular factors like Cullin-5, Elongin B and Elongin C to E1B55K/E4orf6 complex forming a ubiquitin ligase complex (Boyer & Ketner., 2000,Harada *et al*., 2002,Querido *et al*., 2001). The E4orf6 can interact with p53 and p73 independently of E1b55K, and counteract their inhibitory effects on viral replication (Dobner *et al*., 1996,Higashino *et al*., 1998,Steegenga *et al*., 1999). The E4orf6/7 interacts with E2F transcription factors and thus increases their binding to E2 promoter (Neill *et al*., 1990,Schaley *et al*., 2000).

1.1.3.3 Protein-protein interactions of adenovirus intermediate proteins

The adenovirus protein IVa2 is a multifunctional protein and plays a significant role in capsid assembly (Zhang & Imperiale., 2003), packaging of viral DNA (Ahi *et al*., 2015,Zhang *et al*., 2001) and major late promoter activation (Tribouley *et al*., 1994) by interacting with various other adenoviral protein. Earlier studies have reported that IVa2 interacts with A repeats of packaging domain and L152/55k protein suggesting its role in viral DNA encapsidation (Ostapchuk *et al*., 2005,Perez-Romero *et al*., 2005,Zhang *et al*., 2001). Moreover, IVa2 also

interacts with L4 22K and recruits it to packaging domain. It has been suggested that L4 22K also plays an important role in packaging of viral DNA (Ewing *et al*., 2007,Ostapchuk *et al*., 2006,Tyler *et al*., 2007). Since, IVa2 can bind ATP it has been suggested that it can play the role of packaging ATPase. A recent study has reported that ATPase activity of IVa2 is stimulated by viral DNA and L4 33K protein (Ahi *et al*., 2015,Ostapchuk & Hearing., 2008,Ostapchuk *et al*., 2011).

Protein IX a minor capsid protein interacts with PML protein and causes its sequestration and thus counteracts the PML mediated antiviral response during late phase of adenovirus infection (Rosa-Calatrava *et al*., 2003).

1.1.3.4 Protein-protein interactions of adenovirus late proteins

Adenovirus late proteins regulate the late phase translation and assembly of virus particle through their interactions with cellular and other adenovirus protein.

The HAdV-5 L1 52/55K protein interacts with IVa2 protein and also interacts with packaging sequences independent of IVa2 suggesting that it plays a significant role in packaging of viral DNA (Perez-Romero *et al*., 2005). In addition, L1 52/55K interacts with pVII, which appears essential for packaging of viral DNA (Zhang & Arcos., 2005). A recent study has reported that L1 52/55K is cleaved by AVP during the process of virus maturation causing its release from mature virion (Perez-Berna *et al*., 2014).

The HAdV-5 L2 pVII is the most abundant core protein. It binds to the viral DNA and through its interaction with various cellular transport factors like importin and transportin mediates import of viral DNA into nucleus after disassembly of virus particle at NPC (Hindley *et al*., 2007,Wodrich *et al*., 2006,Zhang & Arcos., 2005). It has been reported that pVII bound to adenoviral DNA interacts with cellular protein template activating factor (TAF) 1, which leads to

the release of pVII from viral DNA and stimulation of transcription from viral DNA (Gyurcsik *et al*., 2006). Moreover, pVII interacts with adenovirus packaging proteins IVa2 and L1 52/55K, which may play a vital role in packaging of viral DNA (Zhang & Arcos., 2005).

The HAdV-5 L2 protein V is the second most abundant core protein. Protein V through its interactions with capsid protein pVI and core protein pVII acts as a bridge that connects capsid and the core (Harpst *et al*., 1977,Matthews & Russell., 1998a). The protein V interacts with cellular proteins nucleolin and B23 and causes their redistribution from nucleolus to the cytoplasm (Matthews., 2001). Moreover, protein V interacts with cellular protein p32, which might mediate import of viral DNA from the cytoplasm to the nucleus (Matthews & Russell., 1998b).

During the process of virion maturation, HAdV-5 L3 protein pVI is cleaved by interacting with AVP. The C-terminal 11 amino acids released from pVI then act as cofactor for AVP and cause activation of AVP (Mangel *et al*., 1993,Mangel *et al*., 2003,Webster *et al*., 1993,Webster & Kemp., 1993). Moreover, pVI interacts with hexon capsomeres and appears to mediate their import into the nucleus where assembly of adenovirus virion takes place (Wodrich *et al*., 2003).

The HAdV-5 L4 encoded 100K is a multifunctional protein that interacts with various cellular and other adenovirus proteins, which play a vital role in adenovirus assembly and regulation of late adenoviral protein translation. Earlier studies reported that 100K interacts with hexon, which is essential for trimerization of hexon (Cepko & Sharp., 1982,Oosterom-Dragon & Ginsberg., 1981) and nuclear import of hexon (Hong *et al*., 2005). Moreover, 100K interacts with both tripartite leader and cellular protein eIF4G and these interactions selectively enhances the translation of late adenovirus genes by mechanism of ribosome shunting (Hayes *et al*.,

1990,Xi *et al*., 2004). Another study reported that 100K competes with eIF4E kinase, Mnk1, and displaces it from eIF4G thereby inhibiting cellular mRNA translation (Cuesta *et al*., 2000).

HAdV-5 L4 33 K protein interacts with other viral proteins and through these interactions plays important role in viral DNA packaging and assembly of adenovirus particle. Interaction of 33K protein with IVa2 (Ahi *et al*., 2013) and IIIa (Wu *et al*., 2013) protein have been reported. A recent study has reported that 33K is part of packaging motor and acts similar to small terminase protein (Ahi *et al*., 2015).

HAdV-5 L5 fiber protein interacts with penton base to form penton (Caillet-Boudin., 1989). The knob domain of fiber interacts with cell surface receptors to facilitate attachment of the virus to the cell (Henry *et al*., 1994,Philipson *et al*., 1968).

1.1.4 Bovine adenovirus

Isolation of bovine adenovirus (BAdV) was initially reported in 1959 (Klein *et al*., 1959). So far isolation of 14 different serotypes of bovine adenovirus have been reported (Lehmkuhl & Hobbs., 2008,Sibley *et al*., 2011). BAdVs generally cause mild respiratory or gastrointestinal tract infections in cattle (Lehmkuhl *et al*., 1975,Smyth *et al*., 1996). However, BAdVs have been isolated from seemingly healthy animals (Darbyshire *et al*., 1965).

1.1.4.1 Classification of bovine adenoviruses

Based on various criteria's including genome length, nucleotide content, presence or absence of genus specific antigens and restriction enzyme analysis pattern currently identified BAdVs are classified either under the genus *Mastadenovirus* or the genus *Atadenovirus* (Benkö *et al*., 1988,Lehmkuhl & Hobbs., 2008,Sibley *et al*., 2011).

1.1.4.2 Bovine adenovirus type 3

The isolation of bovine adenovirus 3 (BAdV-3) was first reported from England from the eye of seemingly healthy cattle (Darbyshire *et al*., 1965). Subsequently, its isolation has been reported from cattle showing symptoms of respiratory or gastrointestinal disease (Mattson., 1973a,Mattson., 1973b). Recently, isolation of BAdV-3 has been reported from sick calves in China (Zhu *et al*., 2011). However, studies involving inoculation of calves with BAdV-3 have reported mild or no clinical symptoms in experimentally infected calves (Lehmkuhl *et al*., 1975,Mattson *et al*., 1988,Mittal *et al*., 1999).

1.1.4.2.1 Genome organization

The double stranded DNA genome of BAdV-3 is of 34446 bp in length. The length of BAdV-3 genome and its G+C content (54%) is very similar to that of HAdV-2, HAdV-5 and HAdV-12 (Chroboczek *et al*., 1992,Reddy *et al*., 1998,Sprengel *et al*., 1994). Similar to other adenoviruses BAdV-3 genome is also flanked by inverted terminal repeats (ITRs) (Morikazu *et al*., 1987). However, 195bp long ITRs of BAdV-3 differ from that of HAdV-2 and HAdV-5 by being longer and by having a high G+C content (84%) in the GC-rich region of ITR (Davison *et al*., 2003,Reddy *et al*., 1998). Similar to other adenoviruses the BAdV-3 packaging domain is present at the left end of genome with a number of A repeats located between nucleotide 225 and 310 (Reddy *et al*., 1998). However, a distinct feature of BAdV-3 is that a portion of E1A coding sequence is reported to be essential for BAdV-3 genome packaging. Moreover, it has been reported that the E1A promoter is situated in the left ITR region (Xing & Tikoo., 2006). Similar to other members of the genus *Mastadenovirus*, the genome of BAdV-3 is also structured into early, intermediate and late regions. However, unlike HAdV-5 genome (Chroboczek *et al*., 1992) the late genes of BAdV-3 genome are divided into 7 regions (Fig.1.1.3) (Reddy *et al*., 1998).

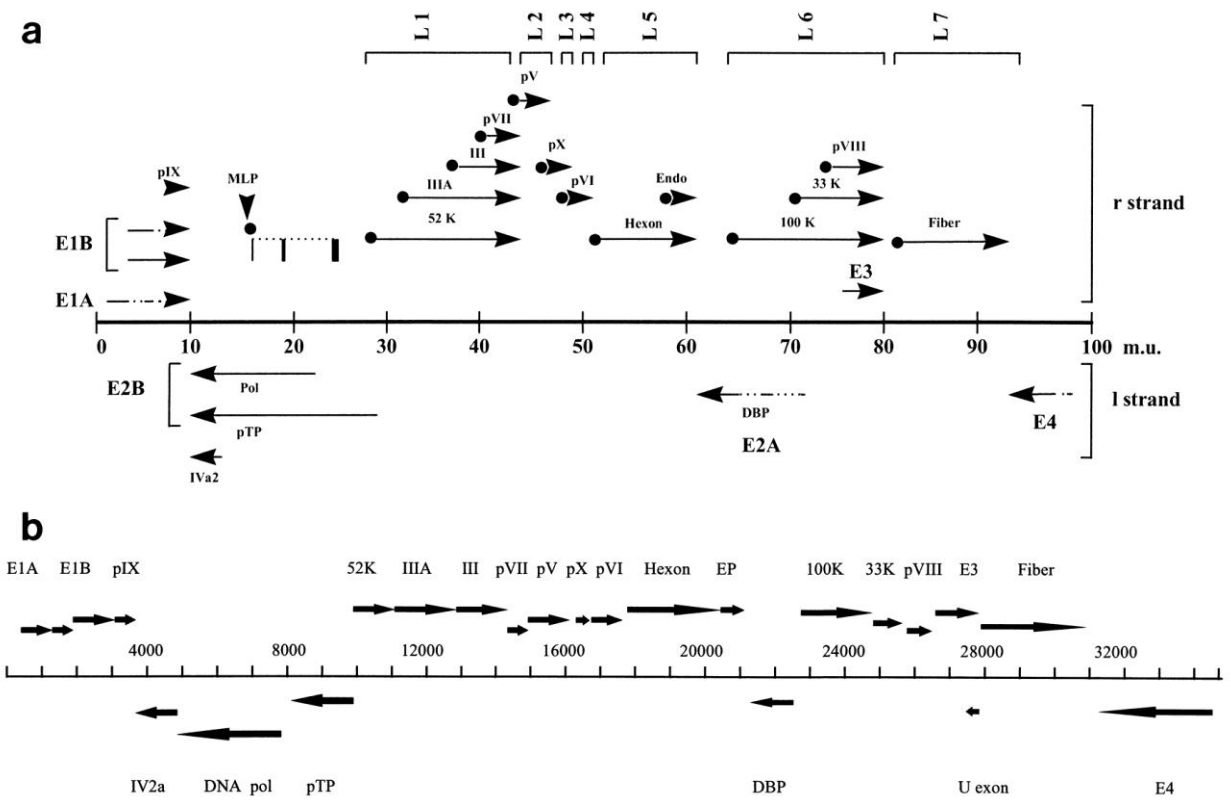


Figure 1.1.3 Schematic representing transcription map and genome organization of BAdV-3. Figure and figure legend reprinted from (Reddy *et al*., 1998) with permission from American Society for Microbiology (publisher). (a) Summary of transcriptional analysis of BAdV-3. The schematic diagram of the BAV-3 genome (34,446 bp) is divided into 100 m.u. Arrows above and below the central line represent mRNAs from the r and l strands, respectively. Solid lines represent sequences found in mRNA, broken lines indicate introns, and arrowheads represent poly (A) sites and show the direction of transcription. Each mRNA in L1 to L7 has a TPL spliced to its 5′ terminus (•). (b) Summary of the genomic organization of BAV-3. Genes are named as homologs of HAV-2. Arrows show the positions of the ORFs for the indicated proteins.

1.1.4.2.2 Early genes

The E1 region of BAdV-3 genome encodes for E1A and E1B. Unlike HAdV (Berk & Sharp., 1978), BAdV-3 E1A and E1B are encoded by a single transcription unit sharing the polyadenylation site (Reddy *et al*., 1999). Using alternative splicing E1A region of BAdV-3 encodes six transcripts that encode three small proteins of 211, 115 and 100 amino acids (Reddy *et al*., 1999). Similar to HAdV, BAdV-3 E1 proteins are necessary for replication of virus and transactivation of other viral genes (Reddy *et al*., 1999,Zheng *et al*., 1994,Zhou *et al*., 2001a) . The E1B region of BAdV-3 is reported to produce two distinct overlapping mRNAs that encodes two different proteins 420R and 157R (Reddy *et al*., 1999). One of the studies has reported that 420R is essential for BAdV-3 replication while requirement of 157R for viral replication is dependent on cell type (Zhou *et al*., 2001a).

The E2 region is divided into two transcriptional units E2A and E2B encoding proteins essential for replication of viral DNA (Reddy *et al*., 1998). The 432 amino acid long DNA binding protein (DBP) is the only protein encoded by E2A. The amino acid sequence of BAdV-3 DBP shows 18 to 47% sequence identities with DBP of other adenoviruses (Reddy *et al*., 1998). Like other adenoviruses, DBP of BAdV-3 also consists of a variable amino-terminal region and a highly conserved carboxy terminal region. The highly conserved carboxy terminal region of HAdV DBP has been reported to be involved in DNA binding and viral replication, and also activation of the MLP (Kitchingman., 1985). Similar to HAdVs, the amino terminal region of BAdV-3 DBP consists of many serine and threonine amino acids, which may function as sites for phosphorylation (Reddy *et al*., 1998). Similar to HAdVs, a number of basic amino acids containing motifs are present in the amino-terminal region of BAdV-3 DBP including ^{29}PRKK^{32},

^{35}RKRR^{38} and ^{53}KRAK^{56}. These potential NLS could serve to import DBP into the nucleus (Reddy *et al*., 1998).

Like HAdVs, the viral DNA polymerase (Pol) protein and the preterminal protein (pTP) are encoded by BAdV-3 E2B region. The BAdV-3 Pol protein consists of 1023 amino acids and shows 59-60% homologies with Pol protein of other members of genus *Mastadenovirus*. The BAdV-3 pTP protein is composed of 649 amino acids and shows 58-60% homologies to pTP protein of other mastadenoviruses (Baxi *et al*., 1998,Reddy *et al*., 1998). It has been reported that a YSRLRYT motif present in pTP is essential for protein primed initiation of DNA replication by pTP (Hsieh *et al*., 1990). The BAdV-3 pTP also contains this motif in form of YSRLVYR (Baxi *et al*., 1998). Unlike HAdV-2 pTP which has three potential protease cleavage sites, BAdV-3 pTP has only two potential protease cleavage sites (Baxi *et al*., 1998,Webster *et al*., 1994).

Compared to other adenoviruses, the 1591 bp long E3 region of BAdV-3 is relatively small. However, similar to other adenoviruses, its location has been mapped to be between the genes coding for pVIII and fiber protein (Idamakanti *et al*., 1999,Reddy *et al*., 1998). The transcripts generated from E3 region code for four proteins with 284 (284R), 121 (121R), 86 (86R) and 82 (82R) amino acids respectively. The 284R protein identified as a glycoprotein does not show any homology to E3 region encoded gp19 glycoproteins of HAdVs (Idamakanti *et al*., 1999). Although, BAdV-3 121R protein shows limited homology with the 14.7K protein of HAdV-5 (Mittal *et al*., 1992), they appear to be functionally similar (Zakhartchouk *et al*., 2001). The E3 region of BAdV-3 is not required for viral replication in cultured cells (Zakhartchouk *et al*., 1998a).

Like other HAdVs, E4 region of BAdV-3 has been mapped to the right end of the genome (Lee *et al*., 1998,Reddy *et al*., 1998). The 3004 bp E4 region of BAdV-3 produces 7 transcripts that encode 5 open reading frames. Out of these five ORFs three ORFs 1, 2 and 4 do not have any homologs in other adenoviruses and thus appear to be unique to BAdV-3. However, ORFs 3 and 5 have been reported to share homology with E4orf6 proteins of HAdVs (Baxi *et al*., 1999,Baxi *et al*., 2001). Mutational analysis of E4 regions has reported that individual ORFs of E4 region are dispensable for viral replication in cultured cells as well as cotton rats (Baxi *et al*., 2001).

The genome of HAdV-2 also contains genes for encoding two non coding RNAs known as virus-associated (VA) RNAs (Reich *et al*., 1966). They counteract the host viral defense mechanism by inhibiting the PKR mediated phosphorylation of eukaryotic initiation factor 2 (eIF2α) (Kitajewski *et al*., 1986,O'Malley *et al*., 1986). Moreover, recent studies have reported that VA RNA inhibits cellular miRNA biogenesis (Andersson *et al*., 2005,Bennasser *et al*., 2011). The location of genes for VA RNAs has been mapped between the 52/55K and pTP encoding genes. However, unlike HAdV-2, so far genes encoding for VA RNAs have not been located in BAdV-3 genome (Reddy *et al*., 1998). Since genes coding for BAdV-3 52/55K and pTP do not have any space between them, it has been speculated that either VA RNA genes are absent from BAdV-3 genome or are located at a different location in genome (Reddy *et al*., 1998).

1.1.4.2.3 Intermediate gene

Similar to HAdVs, BAdV-3 also encodes two intermediate proteins named IVa2 and IX. The BAdV-3 structural protein IVa2 is shorter compared to IVa2 protein of other adenoviruses and is 376 amino acid long (Baxi *et al*., 1998,Morrison *et al*., 1997,Vrati *et al*., 1996). The

BAdV-3 intermediate protein IVa2 shows 29 to 69% identical amino acids when matched to amino acid sequence of IVa2 protein of other adenoviruses (Baxi *et al*., 1998,Reddy *et al*., 1998). In HAdV-2 (Berk & Sharp., 1978) the IVa2 transcript is 3′ co terminal with either pTP or DNA Pol transcripts however, this is not the case with BAdV-3 IVa2. The BAdV-3 intermediate protein IX is shorter than protein IX of HAdV-2 and is 125 amino acid long (Baxi *et al*., 1998,Zheng *et al*., 1994). When compared to amino acid sequence of other adenoviruses protein IX of BAdV-3 shows 16 to 28% similar amino acids (Reddy *et al*., 1998). In BAdV-3 infected cells protein IX can be detected as 14 kDa protein (Reddy *et al*., 1999). Similar to protein IX of other HAdVs (Akalu *et al*., 1999,Dmitriev *et al*., 2002), C-terminus of protein IX of BAdV-3 is also exposed on the surface of viral capsid, which has been exploited for insertion of peptides for targeting of adenovirus vectors to different cells (Akalu *et al*., 1999,Dmitriev *et al*., 2002,Zakhartchouk *et al*., 2004).

1.1.4.2.4 Late genes

Similar to HAdVs, the BAdV-3 MLP is located at 16.2 map units (Reddy *et al*., 1998, Song *et al*., 1996). However, unlike HAdVs in which the late transcription unit gives rise to five families (L1-L5) of late mRNAs (Fraser *et al*., 1982) the BAdV-3 late transcription unit is processed into mRNAs that has been grouped into seven families (L1-L7) based upon usage of polyadenylation sites (Reddy *et al*., 1998). Similar to HAdV-2, BAdV-3 late mRNAs transcribed from MLP contains non coding tripartite leader (TPL) sequence, which in BAdV-3 is 205nt in length. The three parts of TPL are 40nt, 78nt and 87nt in length respectively (Chow *et al*., 1977, Reddy *et al*., 1998).

Unlike HAdVs in which L1 region encodes for only two proteins (52/55K and pIIIa) the L1 region of BAdV-3 encodes for four late proteins 52K, IIIA, III and pVII (Reddy *et al*.,

1998,Sussenbach., 1984).The 52K protein of BAdV-3 is 331 amino acids long and shows 61.6% amino acid identities with its homolog in HAdV-2 (Reddy *et al*., 1998). The BAdV-3 52K is detected as 40kDa protein in infected cells, which localizes predominantly in the nucleus (Paterson *et al*., 2012). The three basic residues ^{105}RKR^{107} of the identified NLS (amino acids 102 to 110) of BAdV-3 52K appear critical for nuclear localization (Paterson *et al*., 2012). Furthermore, BAdV-3 52K interacts with and uses cellular importin α3 of the classical importin α/β pathway for it is transport to the nucleus (Paterson *et al*., 2012). The BAdV-3 52K also interacts with cellular protein NFκB-binding protein (NFBP) and causes its redistribution from the nucleolus to nucleus. The report also suggests that BAdV-3 52K interacts with viral protein pVII (Paterson., 2010).

The 568 amino acid long BAdV-3 IIIa protein shows 25 to 75% amino acid identities to its homologs in other adenoviruses. It has a potential protease cleavage site (LRGT↓G) which is located 19 amino acids away from C-terminus (Reddy *et al*., 1998).

The 482 amino acid long BAdV-3 penton base protein (protein III) shows 44 to 64% identities with amino acid sequences of its homologs in other adenoviruses (Reddy *et al*., 1998). Unlike HAdV-5 (Neumann *et al*., 1988), the penton base protein of BAdV-3 lacks RGD motif, which is required for interacting with integrins (Reddy *et al*., 1998). Moreover, it also lacks the LDV motif present in penton base protein of HAdVs but instead contain a MDV motif (Komoriya *et al*., 1991,Reddy *et al*., 1998). Similar to HAdVs (Caillet-Boudin., 1989), the penton base of BAdV-3 also interacts with fiber protein using the conserved interacting domain located at amino acids 241 to 253 (Caillet-Boudin., 1989,Reddy *et al*., 1998).

The BAdV-3 major core protein pVII is 171 amino acids long and shows 29 to 53% amino acid identities to amino acid sequence of its homologs in other adenoviruses (Morrison *et al*.,

1997,Reddy *et al*., 1998). The BAdV-3 pVII is highly basic and contains a potential adenovirus protease cleavage site (MYGG↓A) located close to its N-terminus (Reddy *et al*., 1998). It has been reported that BAdV-3 pVII localizes to mitochondria, increasing mitochondrial ATP and Ca+ production thus inhibiting apoptosis by preventing the loss of mitochondrial membrane potential (Anand *et al*., 2014).

Unlike L2 region of HAdV-2, which codes for three proteins, the L2 region of BAdV-3 produces a single transcript that encodes protein V (Reddy *et al*., 1998,Sussenbach., 1984). The protein V is unique to the genus *Mastadenovirus (Davison et al., 2003).* The BAdV-3 protein V is 410 amino acids long and shows 28 to 40% amino acid identities to amino acid sequence of protein V of other *Mastadenovirus* (Morrison *et al*., 1997,Reddy *et al*., 1998). Recent report (Zhao., 2016) suggests that BAdV-3 protein V detected as 55kDa protein in infected cells, localizes to both nucleus and nucleolus of infected cells. Furthermore, BAdV-3 protein V contains three nuclear localization signals (amino acids 80-120, 190-210 and 380-389) and preferentially interacts with importin α-3 for its nuclear localization (Zhao., 2016). Moreover, it has been reported that BAdV-3 protein V contains two nucleolar localization signals (NoLs) that have been mapped to amino acids 21-50 and 380-389. Further it has been reported that these NoLs are necessary for viral replication (Zhao., 2016). It has also been reported that protein V is indispensable for viral replication and deletion of protein V from viral genome leads to decrease in viral late protein expression, reduced viral assembly and increased thermolability of progeny virions (Zhao., 2016).

Unlike HAdV-2, the L3 region of BAdV-3 encodes pX protein (Reddy *et al*., 1998,Sussenbach., 1984). Similar to HAdV-2, BAdV-3 pX protein is 80 amino acid long and shows 38 to 64% amino acid identities to amino acid sequence of its homologs in other

adenoviruses (Anderson *et al*., 1989,Reddy *et al*., 1998). The BAdV-3 pX protein is also rich in basic amino acids and contains a bipartite NLS (Anderson *et al*., 1989,Reddy *et al*., 1998). Moreover, BAdV-3 pX also contains 2 potential protease cleavage sites (^{27}LRGG↓S^{31}) and (^{47}LRGG↓F^{51}) (Reddy *et al*., 1998,Russell & Kemp., 1995,Weber., 1995).

Unlike HAdV-2, the L4 region of BAdV-3 encodes pVI protein (Reddy *et al*., 1998,Sussenbach., 1984). The BAdV-3 pVI is 263 amino acids long and shows 15 to 38% amino acid identities to amino acid sequence of its homologs in other adenoviruses (Reddy *et al*., 1998). The BAdV-3 pVI contains two potential adenovirus protease cleavage sites (Reddy *et al*., 1998,Russell & Kemp., 1995,Weber., 1995). Since there is substantial amino acid sequence homology between the C-terminal fragment of pVI protein of HAdV-2 and BAdV-3 it is likely that the C-terminal fragment of BAdV-3 pVI protein may function as cofactor for BAdV-3 protease (Reddy *et al*., 1998).

Unlike HAdV-2, the L5 region of BAdV-3 encodes two proteins namely hexon and protease (Reddy *et al*., 1998,Sussenbach., 1984). The 204 amino acid long BAdV-3 protease shows 30 to 65% amino acid identities with amino acid sequence of its homologs in other adenoviruses (Cai *et al*., 1990,Reddy *et al*., 1998). The potential BAdV-3 protease cleavage sites are conserved in all the precursor proteins (Reddy *et al*., 1998). The major capsid protein hexon is 910 amino acids long in BAdV-3 and shows 45 to 70% amino acid identities to amino acid sequence of hexon protein of other adenoviruses (Hu *et al*., 1984,Reddy *et al*., 1998). In BAdV-3 infected cells hexon can be detected as a 98 kDa protein (Kulshreshtha *et al*., 2004).

The L6 region of BAdV-3 genome encodes three non structural proteins namely 100K, 22K and 33K and one structural protein pVIII (Reddy *et al*., 1998). The 850 amino acid long BAdV-3 100K protein shows 27 to 52% amino acid identities with amino acid sequence of its

homologs in other adenoviruses (Reddy *et al*., 1998). A recent report suggested that BAdV-3 100K acts as a substrate for adenovirus protease and is cleaved at two consensus protease cleavage sites. However, cleavage of BAdV-3 100K protein appears dispensable for virus replication (Makadiya *et al*., 2015). Further, it has been reported that the cleaved C-terminus fragment of 100K contains a nuclear localization signal (amino acids 789-811) which helps to localize the C-terminus of BAdV-3 100K in the nucleus (Makadiya *et al*., 2015). The BAdV-3 100K also interacts with adenovirus protein 33K and cellular protein dynein light chain (DYNLT) 1 (Makadiya., 2013).

BAdV-3 33K and 22K proteins are products of splicing of a single transcript and both share N-terminus 138 amino acids (Kulshreshtha., 2009). Interaction of 33K with adenovirus proteins 100K and protein V and cellular protein bovine presenilin-1-associated protein/mitochondrial carrier homolog 1 (BoPSAP/BoMtch1) has also been reported (Kulshreshtha., 2009,Kulshreshtha & Tikoo., 2008). A recent study has reported that 33K protein uses both importin-α5 and transportin-3 for its nuclear transport. Moreover, it has been reported that conserved arginines of RS repeats that are present at C-terminus of 33K are indispensable for nuclear transport of 33K as well as virus replication (Kulshreshtha *et al*., 2014).

The 274 amino acid long BAdV-3 22K protein is the unspliced form of 33K mRNA. It has been suggested that the 97 amino acids located at N-terminus of 33K/22K may play a vital role in encapsidation of DNA (Kulshreshtha *et al*., 2004).

The hexon associated protein pVIII is the only structural protein that is encoded by the L6 region of BAdV-3. The BAdV-3 pVIII is 216 amino acids long and shows 19 to 56% amino acid identities with amino acid sequence of its homologs in other adenoviruses (Reddy *et al*., 1998,Sussenbach., 1984,Vrati *et al*., 1995). Two potential adenovirus protease cleavage sites

(^{108}IAGG↓G^{112}) and (^{143}LGGG↓S^{147}) are present in pVIII protein of BAdV-3 (Reddy *et al*., 1998).

Unlike HAdV-2, the L7 region of BAdV-3 genome encodes for fiber protein (Reddy *et al*., 1998,Sussenbach., 1984). The 976 amino acid long BAdV-3 fiber protein shows 17 to 26% amino acid identities with amino acid sequence of its homologs in other adenoviruses (Reddy *et al*., 1998). The BAdV-3 fiber protein also contains the conserved hydrophobic sequence motif that is responsible for interacting with penton base protein (Caillet-Boudin., 1989,Reddy *et al*., 1998). Moreover, BAdV-3 fiber protein also contains a conserved TLWT motif at the junction of head and shaft (Chroboczek *et al*., 1995,Reddy *et al*., 1998). Since the tropism of BAdV-3 can be altered by changing the knob region of BAdV-3 fiber protein with knob region of HAdV-5 fiber protein, it is suggested that the knob region of BAdV-3 fiber is responsible for the initial interaction of virus to cellular receptor (Wu & Tikoo., 2004).

1.1.5 Adenovirus protease and its role in adenovirus life cycle

Early studies reported that cleavage of some adenovirus precursor proteins is essential for infectivity of progeny adenovirus (Amin *et al*., 1977,Anderson *et al*., 1973,Edvardsson *et al*., 1976,Weber., 1976). Subsequent studies reported that proteolytic activity observed during adenovirus infection is because of virus encoded protease protein packaged in adenovirus virion (Bhatti & Weber., 1979,Bhatti & Weber., 1978).

Adenovirus protease, a product of L3 transcription unit, has a molecular weight of 23 kDa in HAdV-5. The gene for AVP is highly conserved in all adenoviruses that have been sequenced to date (Davison *et al*., 2003,Webster & Kemp., 1993). Protease protein is packaged into mature virus particle with an estimated copy number ranging between 10 and 50 per virion (Anderson., 1990,Brown *et al*., 1996).

Earlier studies reported that protease activity of adenoviral protease expressed in *E. coli* is inhibited by cysteine protease inhibitor and hypothesized that conserved cysteine residues at position 104 and 122 are crucial for the protease activity (Tihanyi *et al*., 1993). Later analysis of the crystal structure of HAdV-2 protease in complex with C-terminal 11 amino acids of pVI revealed that like papain, adenovirus protease also uses a catalytic triad of H54, E71 and C122 for its activity. These findings lead to the classification of AVP as the earliest member of a novel class of cysteine proteases (Ding *et al*., 1996).

Using synthetic peptides as substrates and by analyzing cleavage of the precursor viral proteins during adenovirus infection, it has been determined that AVP recognizes and cleaves at two consensus cleavage motifs (M/I/L)XGX-G and(M/I/L)XGG-X (where X is any amino acid) (Webster *et al*., 1989). However, some of the studies have reported AVP mediated cleavage at sites other than the consensus cleavage motif suggesting that the specificity requirements for the cleavage motif can be relaxed (Makadiya *et al*., 2015,Perez-Berna *et al*., 2014,Webster *et al*., 1997).

Observations that recombinant AVP produced in bacteria or insect cells has no cleavage activity together with the observation that the protease activity of recombinant AVP can be restored by incubation with disrupted virus led to the conclusion that some adenovirus encoded cofactors are required for AVP activity. Further studies suggested that the C-terminal 11 amino acid of protein pVI and viral DNA act as the cofactors required for the optimal AVP activity (Mangel *et al*., 1993,Webster & Kemp., 1993,Webster *et al*., 1993).

Adenoviral proteins that act as substrate for adenovirus protease include three minor capsid proteins (pIIIa, pVI and pVIII) and three core proteins (pVII, pμ and pTP) (Challberg & Kelly., 1981,Mangel *et al*., 1996,Weber., 1976). Apart from these six structural proteins two

nonstructural proteins L1 52/55k (Perez-Berna *et al*., 2014) and 100K (Makadiya *et al*., 2015) have been recently reported to be the substrates of adenoviral protease. Studies involving a temperature sensitive mutant adenovirus also known as ts1 has provided invaluable information on role of AVP in maturation of adenovirus and its effect on the infectivity (Mangel & San Martín., 2014). The ts1 mutant when propagated at 39°C failed to incorporate AVP in the viral particle. Moreover, though progeny virions contained all AVP target proteins in precursor form, the virions were not infectious. These results suggested that AVP is essential for the maturation and subsequent infectivity of progeny adenovirus (Imelli *et al*., 2009,Rancourt *et al*., 1995,Weber., 1976,Yeh-Kai *et al*., 1983).

Newly synthesized AVP is in its inactive state to protect precursor proteins from being cleaved prematurely. The packaging of AVP in immature particle and its binding to viral DNA leads to its partial activation (Bajpayee *et al*., 2005,Gupta *et al*., 2004,Mangel *et al*., 1993,McGrath *et al*., 2001). When partially activated AVP comes in contact with DNA bound pVI, it cleaves pVI at both N-terminus and C-terminus generating 11 amino acids peptide cofactor pVIc. The released pVIc then binds to AVP bound to DNA leading to its full activation (Baniecki *et al*., 2001,Graziano *et al*., 2013,McGrath *et al*., 2002). Fully activated AVP cleaves six structural proteins and a non structural 52/55k protein causing its release from the virion (Mangel *et al*., 1997,Perez-Berna *et al*., 2014). AVP mediated cleavage of adenoviral proteins thus results in a mature metastable adenovirus particle (Perez-Berna *et al*., 2012).

After the lysis of the infected cells, newly assembled viruses are released into the extracellular environment. The oxidizing conditions of the extracellular environment inactivate the AVP. To infect a new cell, after initial attachment of adenovirus fiber knob with the primary cellular receptor, the penton base interacts with integrins receptors on cell surface and virus

particles are subsequently endocytosed. Interaction of integrins and penton base causes a conformational change that exposes the previously hidden protease cleavage sites of pVI protein (Greber *et al*., 1996). Exposure to reducing environment of endosomes reactivates the AVP and leads to degradation of pVI protein and this starts the uncoating of adenovirus particle. The degradation of pVI protein is blocked and disassembly of virus particle is suspended at nuclear envelop if protease activity is blocked by use of protease inhibitors (Greber., 1998,Greber *et al*., 1996).

It has been reported that late in infection, AVP causes cleavage of cytokeratin 18 at two consensus cleavage sites causing destruction of cytokeratin network of the cell (Chen *et al*., 1993). The actin was identified as a cofactor that can activate AVP in cytoplasm in the absence of other viral proteins (Brown *et al*., 2002). Presence of two AVP consensus cleavage sequences in β-actin suggested that it can also be a substrate for AVP. Incubation of AVP with actin lead to the cleavage of actin at consensus cleavage site confirming that indeed actin acts as a substrate for AVP (Mangel *et al*., 2003). These reports indicated that during the course of adenovirus infection, AVP causes destruction of the cytoskeletal network and thus promotes lysis of adenovirus infected cell, which is essential for the release of progeny virions (Chen *et al*., 1993). Thus, adenovirus protease plays a vital role in maturation, uncoating and release of adenovirus from the infected cells.

1.2 mRNA translation in eukaryotes

mRNA translation in eukaryotes is an essential, multistep, multiprotein, complex process that requires comprehensive biological machinery (Hershey., 1991,Hershey & Merrick., 2000). This elegant process of deciphering a polypeptide sequence from an mRNA transcript can be divided into four stages: translation initiation, elongation, translation termination and recycling

(Kapp & Lorsch., 2004,Sonenberg & Hinnebusch., 2009). In initiation stage, an active 80S ribosome, consisting of 40S and 60S ribosomal subunits, is assembled at the 5′ end of mRNA. The elongation stage follows initiation stage and involves movement of 80S ribosome along mRNA in a 5′ to 3′ direction adding amino acid to polypeptide chain. Termination occurs when a stop codon is encountered in mRNA by the translating ribosome. The newly synthesized polypeptide chain is then released from translating ribosome. In the recycling stage, the 80S ribosome is dissociated into 40S and 60S subunits which can again take part in another round of initiation (Kapp & Lorsch., 2004). Since protein synthesis is central to the cell survival and well being a large proportion of cellular resources and energy is devoted towards translation and its regulation. Although all stages of translation are tightly regulated, the regulation of translation initiation is most critical (Mathews *et al.*, 2007,Preiss & W Hentze., 2003,Sonenberg & Hinnebusch., 2009).

1.2.1 Translation initiation

Translation initiation is the process of assembly of protein synthesis machinery on an mRNA transcript and involves structural elements of mRNA and several coordinated mRNA-protein and protein-protein interactions (Preiss & W Hentze., 2003). A number of proteins that play important role in translation initiation are present in limiting amounts in cells, therefore translation initiation is the critical step for protein synthesis (Preiss & W Hentze., 2003,Sachs & Varani., 2000).

1.2.1.1 Structural elements of eukaryotic mRNA

Since cellular nucleases can easily access and target the 5′ and 3′ ends of newly synthesized mRNA transcript, the ends of mRNA must be protected from this nuclease mediated degradation. This is accomplished by addition of a 5′ 7-methylguanosine cap structure at time of

transcription and a 50-300 nucleotide long 3′ poly A tail immediately after transcription (Hall., 2002,Schoenberg & Maquat., 2009,Wilusz *et al*., 2001). These structural elements not only protect the mRNA transcript but also regulate processing, export from nucleus to cytoplasm and translation of mRNA transcript (Wilusz *et al*., 2001).

The 5′ cap structure plays an important role in regulating the important features of mRNA biology including RNA splicing, export from nucleus and translation (López-Lastra *et al*., 2010). During the process of translation initiation, the cap structure through its interaction with eukaryotic initiation factor (eIF) 4E recruits the 40S ribosomal subunit to the mRNA transcript (described in section 1.2.1.2).

The presence of a poly (A) tail at the 3′ end of mRNA transcript is also important for providing stability to mRNA transcript. Except histone mRNAs, majority of the mammalian mRNA transcripts have a poly (A) tail ranging from 50-300 nucleotides long (Dominski & Marzluff., 2007). Poly (A) - binding protein (PABP), which has high affinity for polyadenylated mRNA, binds to poly A tail (Mangus *et al*., 2003). Moreover, PABP also interacts with 5′ end bound eIF4G leading to the circularisation of mRNA transcript. This circular conformation increases the stability of mRNA transcript and protects 3′ and 5′ ends of mRNA by blocking the access to nucleases and decapping enzymes (Amrani *et al*., 2008,Sachs & Varani., 2000).

1.2.1.2 Cap dependent translation initiation

Translation initiation is a process involving a number of steps that require orderly interactions of at least 12 well defined initiation factors (Pestova *et al*., 2007). Since, translation initiation necessitates dissociated ribosomal subunits, the first step in the initiation process is building the pool of 40S and 60S ribosomal subunits. This is accomplished by blocking physiologically favourable joining of dissociated 40S and 60S subunits by tight binding of three

initiation factors eIF1A, eIF3 and eIF6 to dissociated subunits (Jackson *et al*., 2010,Pestova *et al*., 2007). eIF6 interacts with 60S subunit (Valenzuela *et al*., 1982) while eIF1A and eIF3 bind tightly to 40S subunit (Thomas *et al*., 1981). Once a pool of 40S subunit is established, the next step is the recruitment of initiator tRNA, Met-tRNAi and formation of a ternary complex consisting of eIF2, GTP and Met-tRNAi. The eIF2 first binds GTP and subsequently eIF2-GTP complex binds Met-tRNAi to produce the ternary complex (Erickson & Hannig., 1996,Kapp & Lorsch., 2004). The ternary complex then interacts with 40S subunit to form the 43S preinitiation complex (PC). The 40S subunit bound factors eIF1, eIF1A and eIF3 facilitate interaction of the ternary complex and the 40S subunit (Majumdar *et al*., 2003,Trachsel *et al*., 1977).

The 43S PC is then recruited to the mRNA using the 5′ cap structure of the mRNA as a recruitment site. This recruitment of the 43S PC to the 5′cap is facilitated by the eIF4F complex. The eIF4F is a multiprotein complex consisting of cap binding protein eIF4E, RNA helicase eIF4A and the scaffolding protein eIF4G (Pestova *et al*., 2007).

The cap binding protein eIF4E has a baseball glove like structure made up of eight stranded anti parallel beta sheets and three long helices. The concave region of the eIF4E formed between two tryptophan residues provide binding site for the cap structure of mRNA whereas convex surface of the protein provides binding site for eIF4G (Marcotrigiano *et al*., 1997,Matsuo *et al*., 1997,von der Haar *et al*., 2004). Once eIF4E binds to the cap structure of a mRNA, it facilitates the interaction of eIF4A and eIF4G to the 5′ end of mRNA (Pestova *et al*., 2007).

The eIF4A, a protein that belongs to DEAD-box protein family, is a 46 kDa protein with both RNA helicase and RNA dependent ATPase activity. Although, eIF4A has a weak inherent helicase activity, its activity is significantly enhanced by eIF4B and by incorporation into the eIF4F complex (Rogers *et al*., 2001,Rozen *et al*., 1990). It has been suggested that the increased

helicase activity of eF4A-eIF4B complex plays a role in unwinding complex secondary structures in the 5′ UTR of mRNA and aids in recruitment of 43S PC (Rogers *et al*., 1999,Sonenberg., 1996).

The 176 KDa eIF4G is the largest member of eIF4F complex. It binds to eIF4E, eIF4A, eIF3 and PABP and thus acts as a scaffold for the formation of an active translation initiation complex (Keiper *et al*., 1999,Prévôt *et al*., 2003). The attachment site for eIF4E is present at the N-terminus whereas the middle and C-terminal presents site for eIF4A binding (Imataka & Sonenberg., 1997,Mader *et al*., 1995). Because of its binding with eIF3 (part of 43S PC), the eIF4G acts as a bridge between mRNA and 40S ribosome (Gingras *et al*., 1999,Sachs *et al*., 1997). Moreover, by binding to PABP, the eIF4G links 3′ poly A tail and 5′cap structure and thus causes circularization of mRNA (Kahvejian *et al*., 2001).

Once 43S PC is positioned near mRNA, it moves in 5′ to 3′ direction along the mRNA scanning for the AUG initiation codon (Kozak., 1978). The first AUG sequence in an optimal sequence context for initiation (GCC(G/A)CCAUG) is the initiation codon (Kozak., 1986,Kozak., 1987). Once an appropriate initiation codon is identified, the base pairing of the AUG initiation codon and the CAU anticodon of Met-tRNAi leads to the production of 48S initiation complex (Pestova *et al*., 1998). After formation of 48S initiation complex the 40S bound initiation factors (eIF1A and eIF3) are removed from 40S subunit to allow joining of 60S subunit. This is facilitated by eIF5, which causes hydrolysis of GTP in ternary complex eIF2-GTP-Met-tRNAi that leads to the release of eIF2-GDP. This is followed by release of eIF3 from 48S complex. Subsequently, eIF5B mediates the release of eIF1, eIF1A, remaining eIF2-GDP and joining of 60S subunit resulting in generation of functional 80S ribosome (Pestova *et al*., 2000,Unbehaun *et al*., 2004).

1.2.1.3 Cap independent translation initiation

The majority of protein synthesis in the eukaryotic cells is reliant on the cap structure present at 5′ terminus of mRNA. However, some cellular and viral RNAs do not have a cap structure and are translated by an alternative mechanism that involves 5′ untranslated region (UTR) that have extensive secondary structure, known as internal ribosome entry site (IRES) (Hellen & Sarnow., 2001). The first IRES sequences were discovered in picornavirus mRNAs (Jang *et al*., 1988,Pelletier & Sonenberg., 1988) and later on IRES sequence was discovered in a cellular mRNA (Macejak & Sarnow., 1991). Subsequently, the IRES sequence has been found in a number of cellular mRNAs including oncogene c-myc, fibroblast growth factor-2 (FGF-2), eIF4GII, heat shock protein 70 (HSP 70), and X-linked inhibitor of apoptosis protein (XIAP) (Byrd *et al*., 2005,Holcik *et al*., 1999,Rubtsova *et al*., 2003,Stoneley *et al*., 2000,Vagner *et al*., 1995). The mRNAs that contain IRES sequences are normally expressed at low levels, however; they are expressed more efficiently if cap dependent translation is inhibited or down regulated (i.e. due to stress or viral infection) (Merrick., 2004,Qin & Sarnow., 2004). A number of viruses including coronaviruses, flaviviruses, picornaviruses, retroviruses and herpesviruses have been reported to contain IRES sequence in their mRNA (López-Lastra *et al*., 2010).

1.2.2 Viral inhibition of cellular mRNA translation

Viruses do not have their own metabolic machinery so they depend completely on their host for translation of their transcripts. Viruses must therefore compete with host cell for protein synthesis machinery. However, it is difficult for the virus to compete because viral transcripts are outnumbered by large number of cellular transcripts (Mohr *et al*., 2007,Schneider & Mohr., 2003). The majority of protein synthesis in the eukaryotic cells is reliant on the cap structure present at 5′ terminus of mRNA. This absolute dependence of host cellular mRNA on cap

initiation complex has provided viruses with the unique opportunity to compete with host. The easiest way for the virus to acquire host protein synthesis machinery is to inhibit host protein synthesis by targeting the process of cap dependent translation and at the same time develop specific translation mechanisms for itself that bypasses this requirement. Thus, viruses have developed sophisticated alternative means for mRNA translation. These specialized mechanisms reduce their dependence on cap dependent translation or provide them an edge for competing with the cellular transcripts. Many viruses globally interfere with the host translation by inhibiting cap dependent ribosome recruitment to host mRNA (Firth & Brierley., 2012,Gale *et al*., 2000). Viruses can target initiation, elongation or termination stage of translation (Walsh & Mohr., 2011). Since translation initiation is the most critical step for eukaryotic translation, most of the viruses target the translation initiation factors to inhibit cap dependent translation (Gale *et al*., 2000).

1.2.2.1 Inhibition of protein synthesis by targeting eIF4F

The eIF4F is a multisubunit complex formed by three important proteins i) eIF4G-the scaffold protein, ii) eIF4E- the cap binding protein and iii) eIF4A-the RNA helicase (Gingras *et al*., 1999) The eIF4F proteins perform several key functions at the time of cap dependent translation in eukaryotes such as recognition of the 5′cap, recruitment of 40S ribosomal subunit and unwinding of RNA secondary structure. Thus, it is not unexpected that several viruses target eIF4F to shut off the host protein synthesis by making eIF4F subunit non functional or by altering the eIF4F binding proteins (Walsh & Mohr., 2011).

1.2.2.1.1 Targeting of eIF4G by viral proteases

The eIF4G acts as a scaffold protein and interacts with several proteins essential for the recruitment of the ribosome to 5′ end of mRNA. It interacts with eIF4E, eIF3, PABP and the

MAPK integrating kinase (Mnk), for forming an active translation initiation complex (Prévôt *et al*., 2003). Because of its central role in translation initiation, several viruses encode proteases to target eIF4G. Poliovirus, rhinovirus and retroviruses cleave eIF4G by using virus encoded proteases (Etchison *et al*., 1982,Haghighat *et al*., 1996,Ventoso *et al*., 2001). This cleavage of eIF4G by viral proteases separates the eIF4E binding amino terminal region from eIF3 and eIF4A associated carboxy terminal fragment. This prevents formation of a functionally active eIF4F complex, thus inhibiting the cap dependent cellular proteins synthesis. This inhibition of host cap dependent protein synthesis does not inhibit viral mRNA translation because the viral mRNA of these viruses contains IRES sequences that mediate cap independent protein synthesis (Walsh & Mohr., 2011).

1.2.2.1.2 Modulation of eIF4E availability

The eIF4E protein is a key translation initiation factor. The eIF4E binds to the 5′ cap structure of an mRNA, eIF4A and eIF4G. In comparison to other translation factors, eIF4E is present in limited quantities in the cell, which helps to regulate formation of eIF4F complex by controlling availability of eIF4E by 4E binding proteins (4EBP)(Matsuo *et al*., 1997,Sonenberg., 1996). The 4EBP's are low molecular weight proteins, which can act as translation repressors by preventing the formation of eIF4F complex. The 4EBP's binds tightly to eIF4E in the same region where eIF4G binds to eIF4E (Gingras *et al*., 1999,Gingras *et al*., 2001b). The interaction of 4EBP and eIF4E is regulated by phosphorylation of 4EBP's. In their hypophosporylated form, the 4EBP's binds to eIF4E, eliminating its binding to eIF4G and hence inhibiting the cap dependent translation. The phosphorylation of 4EBP reduces its affinity for eIF4E and hence increasing the availability of eIF4E for incorporation into eIF4F (Gingras *et al*., 2001a,Lin *et al*., 1994,Svitkin *et al*., 2005). Several viruses target this process for inhibiting cap dependent

translation. Hypophosphorylation of 4EBP in encephalomyocarditis virus (EMCV) infected cell leads to inhibition of cellular protein synthesis (Gingras *et al*., 1996). Viral mRNA translation is not inhibited because of IRES elements that are able to recruit ribosomes in absence of a cap and eIF4E. This mechanism of inhibition of cellular protein synthesis is also seen during vesicular stomatitis virus (VSV) where Akt-signaling is suppressed by viral M protein leading to prevention of mTORC1 facilitated inactivation of 4EBP1 (Dunn & Connor., 2011). Similarly,In SV40 infection, small T-antigen associates with cellular protein phospahatase 2A (PP2A) leading to dephosphorylation of 4EBP1 (Yu *et al*., 2005). The accumulation of hypophosphorylated 4EBP to inhibit eIF4F assembly is also demonstrated in Dengue virus, Sindbis virus and cricket paralysis virus infected cells (Garrey *et al*., 2010,Mohankumar *et al*., 2011,Villas-Bôas *et al*., 2009).

Interestingly, enterovirus infection leads to increased production of cellular miRNA mir-141 which decreases the expression of its target gene eIF4E thus resulting in inhibition of cap dependent protein synthesis (Ho *et al*., 2011).

1.2.2.1.3 Modulation of eIF4E activity by phosphorylation

The eIF4G associated kinases Mnk1 and Mnk2 causes phosphorylation of eIF4E. However, effect of Mnk1 and Mnk2 mediated phosphorylation of eIF4E on translation initiation is not clear (Pyronnet *et al*., 1999,Scheper & Proud., 2002,Scheper *et al*., 2001). Reduction in the level of phosphorylation of eIF4E has been suggested to be responsible for inhibition of mRNA translation in mammalian cells infected with several viruses including adenovirus and influenza virus (Cuesta *et al*., 2000,Cuesta *et al*., 2004,Feigenblum & Schneider., 1993). The cellular protein synthesis is inhibited at late time in adenovirus infected cell because of virus induced under phosphorylation of eIF4E. Adenoviral 100K protein binds to C-terminus of eIF4G and

prevents binding of Mnk1 to eIF4G thereby blocking phosphorylation of eIF4E (Cuesta *et al*., 2004).

1.2.2.2 Inhibition of protein synthesis by targeting Poly (A) binding protein

The PABP1 is an essential protein and plays a vital role in mRNA stability and translation initiation. The poly (A) tail of majority of the transcripts is covered with multiple copies of PABP1 (Gorgoni & Gray., 2004,Mangus *et al*., 2003). The PABP interacts with eIF4G bridging 5′ and 3′ termini of mRNA causing circularization of the translation initiation complex. This circulariztion of translation initiation complex increases efficiency of translation by stabilizing assembled initiation factors (Sachs *et al*., 1997,Svitkin *et al*., 2001). Several viruses target PABP and disrupt its function. In enteroviruses or coxsackievirus infected cells, the PABP is cleaved by viral encoded protease 2A (Joachims *et al*., 1999,Kerekatte *et al*., 1999). Similarly, in poliovirus infected cells, the PABP is cleaved by virus encoded protease 3C (Kuyumcu-Martinez *et al*., 2004b). However, in calcivirus infected cells, the virus encoded 3C like protease cleaves PABP (Kuyumcu-Martinez *et al*., 2004a). The Inhibition of host cap mediated translation in rotavirus infected cells occurs by blocking the function of PABP. Rotavirus mRNA's have a 5′cap structure but lack 3′ poly (A) tail. The 3′ untranslated region of rotavirus mRNA contain a conserved tetranucleotide sequence, which is recognized by viral protein NSP3. The NSP3 also binds to eIF4G through its C- terminal domain and assumes the role of PABP in translation of rotavirus mRNA. Moreover, eIF4G has more affinity for viral NSP3 than for PABP. This leads to the disruption of interaction between eIF4G and PABP leading to reduced efficiency of host mRNA translation (Groft & Burley., 2002,Piron *et al*., 1998,Vende *et al*., 2000)

1.2.2.3 Inhibition of protein synthesis by altering the distribution of translation initiation factors

Several viruses inhibit protein synthesis by altering the local abundance of translation initiation factors. Vaccinia virus and African swine fever virus replicate in dedicated compartments in cytoplasm. They cause redistribution of eIF4E and eIF4G to these dedicated compartments (Castelló *et al*., 2009,Katsafanas & Moss., 2007,Walsh *et al*., 2008). Poliovirus causes redistribution of eIF4E (Sukarieh *et al*., 2010). Several viruses including bunyavirus, rotavirus, herpes simplex virus (HSV)-1 and Kaposi sarcoma herpes virus (KSHV) cause redistribution of PABP to the nucleus (Blakqori *et al*., 2009,Clyde & Glaunsinger., 2010,Harb *et al*., 2008,Salaun *et al*., 2010).

1.2.2.4 Inhibition of protein synthesis by targeting mRNA

Several viruses target 5′ Cap structure of host mRNA to interfere with cellular protein synthesis. Influenza virus and hantavirus encode a viral endonuclease to steal caps along with 10-18 nucleotide segment from host mRNA (Mir & Panganiban., 2008,Mir *et al*., 2008,Plotch *et al*., 1981). Poxviruses and asfarviruses produce decapping enzyme that remove the 5′ cap from host mRNA resulting in the inhibition of cap dependent protein synthesis (Parrish *et al*., 2007,Parrish & Moss., 2007).

1.2.3 Eukaryotic initiation factor 6 (eIF6)

Eukaryotic translation initiation factor 6 (eIF6) is an important protein that is necessary for 60S ribosome biogenesis and assembly (Basu *et al*., 2001,Valenzuela *et al*., 1982). It was initially isolated independently from both wheat germ and calf liver extract (Russell & Spremulli., 1979,Valenzuela *et al*., 1982). The eIF6 is highly conserved from yeast to mammals (Biffo *et al*., 1997). The human eIF6 is 245 amino acids long and shows 77% identity to its

homolog in yeast cells (Si *et al*., 1997,Si & Maitra., 1999). The eIF6 is expressed as 25 kDa protein at varying levels within the tissues. The brain and epithelia have high levels of eIF6 expression while muscles show low level of eIF6 expression. Within an organ, eIF6 expression is high in stem cells and dividing cells (Donadini *et al*., 2001). However, it is still not known how expression of eIF6 is regulated at molecular level.

Earlier studies involving yeast cells reported that depletion of Tif6 (yeast eIF6 homologue) results in rapid reduction of 60S ribosomal subunits compared to 40S subunit. This effect could be reversed if human eIF6 is supplemented (Sanvito *et al*., 1999,Si & Maitra., 1999,Wood *et al*., 1999) indicating that eIF6 plays an important role in ribosome biogenesis. However, there is no study that can provide a direct evidence for role of eIF6 in ribosome biogenesis in mammalian cells (Miluzio *et al*., 2009).

It was found that eIF6 can bind to immature 60S subunit, which prevents joining of 40S ribosomal subunit with 60S subunits and thus 80S initiation complex is not formed (Valenzuela *et al*., 1982). Since translation initiation necessitates availability of separated 40S and 60S ribosomal subunits, and eIF6 because of its ability to prevent joining of 40S and 60S ribosomal subunit was thought to play a major role in providing a pool of separated ribosomal subunits, it was classified as a translation initiation factor (Biswas *et al*., 2012). For initiation of translation, the release of eIF6 from 60S subunit is essential (Ceci *et al*., 2003). Recent studies have reported that 40S and eIF6 bind to same site of 60S and thus presence of eIF6 on 60S subunit presents a stearic hindrance for 40S subunit thus modulating the joining of 40S to 60S subunit (Gartmann *et al*., 2010,Klinge *et al*., 2011).

The eIF6 is localized in the nucleus as well as in the cytoplasm of both mammalian and yeast cells (Horsey *et al*., 2004,Lebreton *et al*., 2006,Sanvito *et al*., 1999). eIF6 is found bound

to immature 60S ribosomal subunit in the nucleolus. During pre 60S maturation, eIF6 is found in the nucleus and it then moves to the cytoplasm along with pre 60S ribosomal subunit. After maturation of 60S subunit in cytoplam, eIF6 is released from the mature 60S subunit in the cytoplasm, which allows joining of 60S and 40S subunits leading to the formation of functional 80S ribosome (Fig. 1.2.1) (Ceci *et al*., 2003,Horsey *et al*., 2004,Miluzio *et al*., 2009,Si & Maitra., 1999).

It has been reported that eIF6 is a nucleo-cytoplasmic shuttling protein, which is regulated by phosphorylation and dephosphorylation of eIF6 (Basu *et al*., 2003,Biswas *et al*., 2011). The CK1 mediated phosphorylation of Ser-174 and Ser-175 residues of pre-60S bound eIF6 are essential for its export from the nucleus (Basu *et al*., 2003). Moreover, following its release from 60S ribosomal subunit in the cytoplasm, the phosphorylated eIF6 is dephosphorylated by calcium dependent phosphatase calcineurin and this dephosphorylated form of eIF6 is imported back into the nucleus by some yet unknown mechanism (Biswas *et al*., 2011).

The mechanism of release of eIF6 from 60S subunit is still not clear and two different mechanisms have been proposed. First, protein kinase C (PKC)-RACK1 (receptor for activated kinase C) complex in mammalian cells mediates phosphorylation of eIF6 at Ser-235 that leads to the release of eIF6 from 60S subunit (Ceci *et al*., 2003). However, studies involving yeast cell reported that release of eIF6 from 60S subunit is caused by co-operative action of GTPase elongation factor-like1 (Efl 1 p) and Sdo1 (yeast orthologue of mammalian Shwachman-Bodian-

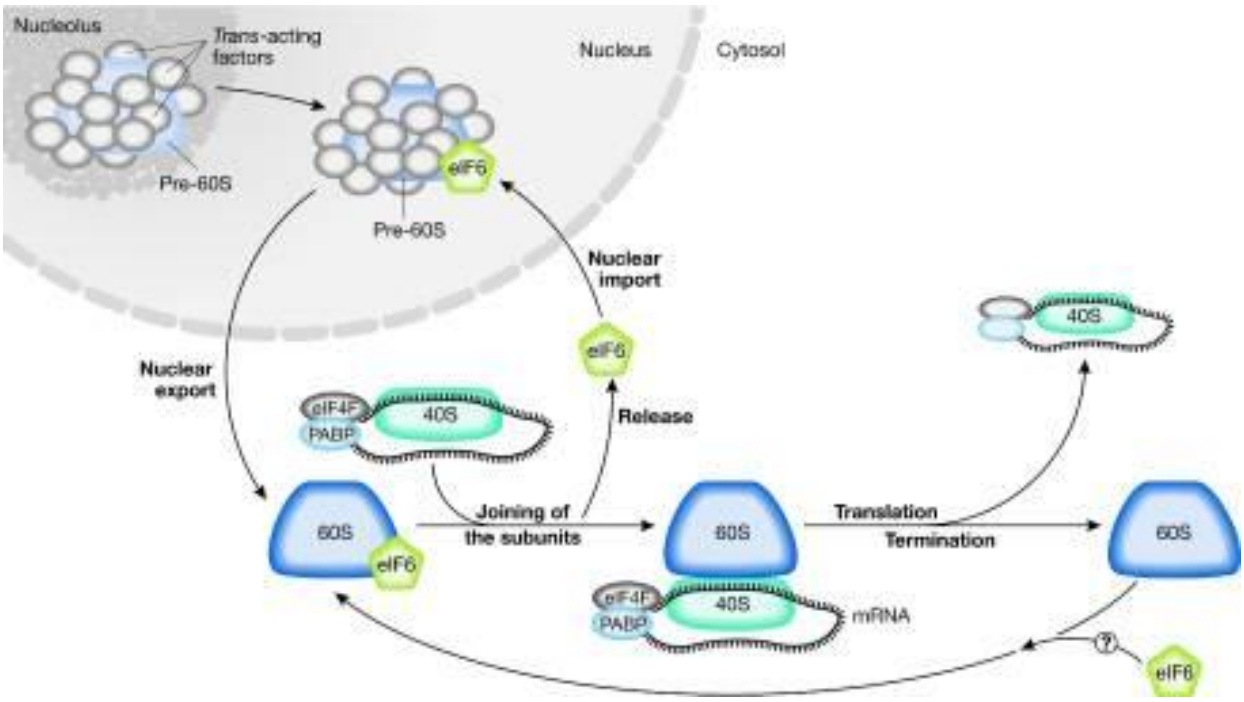


Figure 1.2.1 Nucleocytoplasmic shuttling of eIF6 and its anti-association activity. Reprinted from (Miluzio *et al*., 2009) with permission from John Wiley and Sons (publisher).

Diamond syndrome protein) (Bécam *et al*., 2001,Menne *et al*., 2007,Senger *et al*., 2001). One of the recent *in vitro* studies have reported that purified recombinant SBDS and EFL1 proteins when incubated with eIF6 bound 60S subunits can cause release of eIF6 from the 60S subunit (Finch *et al*., 2011). A recent cryo –EM study have suggested that binding of EFL1 to 60S ribosome causes repositioning of SBDS on 60S ribosome, which facilitates a conformational change in EFL1. The EFL1 then compete with eIF6 for a corresponding binding site on 60S ribosome causing release of eIF6 from 60S ribosome (Weis *et al*., 2015).

A number of studies have tried to identify the role of eIF6 in cancer biology. The eIF6 expression has been found to be upregulated in certain cancer types including colorectal cancer, lung metastasis, head and neck cancer, malignant mesothelioma and acute promyelocytic leukemia. However, no study has been able to establish the etiologic role of eIF6 in cancer (Biffo *et al*., 1997,Harris *et al*., 2004,Martín *et al*., 2008,Rosso *et al*., 2004,Sanvito *et al*., 2000).

1.1.6 The polypeptide pVIII

The polypeptide pVIII is one of the minor capsid proteins that is present at the inner surface of the capsid and connects capsid to the core (Reddy *et al*., 1998,Rohn *et al*., 1997). The polypeptide pVIII is produced and present in immature capsid in precursor form (Takahashi *et al*., 2006, Vellekamp *et al*., 2001). However, only the cleaved fragments of pVIII can be detected in mature virus particles (Vellekamp *et al*., 2001).

The pVIII encoded by L4 region of HAdV-5 is 227 amino acids long (Chroboczek *et al*., 1992) and contains three potential adenovirus protease cleavage sites (Diouri *et al*., 1996). Earlier mass spectrometry studies involving purified HAdV-5 virions reported presence of amino- terminal 112 amino acids (Liu *et al*., 2003), carboxy-terminal 70 amino acids (Takahashi *et al*., 2006) or both amino-terminal 112 amino acids and carboxy-terminal 70 amino acids of

pVIII in mature virions (Blanche *et al*., 2001,Chelius *et al*., 2002). However, a cryoEM study has confirmed the presence of both amino-terminus (112 amino acids) and carboxy-terminus (70 amino acids) fragments of pVIII in mature virus particles (Liu *et al*., 2010). Further studies have reported that two structurally different copies of pVIII (named A and B) bind to the hexons. The VIII-A is a member of V-VI-VIII ternary complex. This ternary complex provides stability to peripentonal hexons while VIII-B is a part of a binary complex VIII-B- VIB that contributes to stabilization of group of nine hexons (GONs) (Reddy & Nemerow., 2014). Thus, localization of polypeptide VIII points to its role in the stabilization of virion particle. One of the studies has reported that mutation in pVIII affects the stability of the adenovirus and makes them more thermolabile (Liu *et al*., 1985).

The BAdV-3 pVIII encoded by the L6 region of the genome is 216 amino acids long and shows 19 to 56% amino acid identities with amino acid sequence of its homologs in other adenoviruses (Reddy *et al*., 1998,Sussenbach., 1984). Two potential adenovirus protease cleavage sites [(^{108}IAGG↓G^{112}) and (^{143}LGGG↓S^{147})] are present in pVIII protein of BAdV-3. Recent report (Ayalew., 2015) suggest that BAdV-3 pVIII detected as 24kDa protein in infected cells localizes to both nucleus and cytoplasm of infected cells. Moreover, BAdV-3 pVIII contains a nuclear localization signal (amino acids 57-72) and preferentially interacts with importin α-3 for its nuclear import (Ayalew *et al*., 2014). Moreover, it has been reported that BAdV-3 pVIII interacts with cellular DEAD-box protein 3 (DDX3) and this interaction leads to inhibition of cap dependent mRNA translation (Ayalew., 2015).

2.0 HYPOTHESIS AND OBJECTIVES

Although adenoviruses have been studied extensively, there is still paucity of information regarding the role of structural proteins in the infectious cycle particularly, with respect to the interaction between viral structural proteins and cellular factors. Among all the adenovirus structural proteins, minor capsid protein pVIII is least characterized (San Martín., 2012). The L4 region encoded HAdV-2 pVIII (Chroboczek *et al*., 1992) connects the inner surface of the capsid to core and plays a significant role in providing stability to the virion.

Unlike HAdV-pVIII, BAdV-3 pVIII encoded by the L6 region of the genome is 216 amino acids long. Two potential adenovirus cleavage sites (^{108}IAGG↓G^{112}) and (^{143}LGGG↓S^{147}) are present in pVIII protein of BAdV-3 (Reddy *et al*., 1998). Apart from pVIII, other adenoviral structural proteins that are substrate for adenovirus protease include minor capsid proteins pIIIa and pVI and core proteins pVII, pμ and pTP (Challberg & Kelly., 1981,Mangel *et al*., 1996,Weber., 1976). Adenovirus protease mediated cleavage of these proteins is essential for maturation and subsequent infectivity of adenovirus. However, importance of each independent cleavage in virion stability and infectivity is still lacking. Moreover, the role of pVIII and its cleavage in the life cycle of adenovirus is still not clear. To understand the role of cleavage of each precursor protein, mutational studies involving mitigation of cleavage site are required.

Viral proteins interact with host cellular proteins to create a favorable environment for virus multiplication. To understand the basic biology of a virus it is necessary to study these virus host interactions. Results of our recent yeast two hybrid experiments indicated that BAdV-3 pVIII interacts with cellular protein eIF6. The eIF6 is a highly conserved protein that is essential for ribosome biogenesis and assembly and possesses a unique anti-association activity that keeps immature 60S and 40S ribosome separated (Miluzio *et al*., 2009).

Taking all these points in consideration we hypothesize that pVIII of BAdV-3 is a multifunctional protein that interacts with cellular protein eIF6 and adenovirus protein protease and through these interactions play an important role in virus replication, virus assembly, stability and infection.

The specific objectives of this work are:

1. Confirmation of interaction of pVIII with eIF6
2. Identification of interacting region of pVIII and eIF6
3. Determining the biological significance of interaction of pVIII and eIF6
4. Study the role of cleavage of pVIII in adenovirus life cycle

3.0 BOVINE ADENOVIRUS-3 pVIII ASSOCIATES WITH EUKARYOTIC INITIATION FACTOR-6 DURING INFECTION

3.1 Introduction

Adenoviruses are large, nonenveloped viruses that have linear double stranded DNA (26 to 45kb) as their genome (Davison *et al*., 2003) enclosed in an icosahedral capsid (Berk., 2013). Adenoviruses are members of family *Adenoviridae* which is further divided into five genera: *Mastadenovirus, Aviadenovirus, Ataadenovirus, Siadenovirus* and *Ichtadenovirus.* Members of *Mastadenovirus* genus including bovine adenovirus (BAdV) -3 encode genus specific protein named pVIII, a structural protein associated with hexon connecting the core with the adenovirus capsid (Reddy *et al*., 1998,Russell., 2009).

Viruses depend absolutely on translational machinery of their host cells for production of various proteins essential for their replication and progeny production. Viruses must therefore compete with cellular mRNAs for limited translation factors that are present in the eukaryotic cells. The majority of protein synthesis in eukaryotic cells is reliant on cap structure present at 5′ terminus of mRNA. This absolute reliance of host cellular mRNA on cap initiation complex has provided viruses with excellent opportunity to develop sophisticated translation mechanism that help them circumvent this requirement (Gale *et al*., 2000,Smith & Gray., 2010,Walsh *et al*., 2013). Although a number of mechanisms have been reported for selective translation of viral mRNAs (Firth & Brierley., 2012,López-Lastra *et al*., 2010,Mohr *et al*., 2007,Walsh & Mohr., 2011), most of the viruses target translation initiation factors to inhibit cap dependent translation and then use the resources and host translation machinery for synthesis of their own proteins (Gale *et al*., 2000,López-Lastra *et al*., 2010,Schneider & Mohr., 2003,Smith & Gray., 2010,Walsh & Mohr., 2011,Walsh *et al*., 2013).

Eukaryotic initiation factor 6 (eIF6) is one of the important initiation factors that plays an important role both in ribosome biogenesis and protein translation. It is a 25 kDa nucleocytoplasmic shuttling protein that is present bound to immature 60S unit in the nucleolus and the nucleus. In the cytoplasm, it is bound to free 60S ribosomal subunit (Horsey *et al*., 2004,Russell & Spremulli., 1979,Valenzuela *et al*., 1982). The nucleocytoplasmic shuttling of eIF6 is controlled by its dephosphorylation and phosphorylation by cellular kinases (Biswas *et al*., 2011,Ceci *et al*., 2003). The presence of eIF6 on the 60S ribosomal subunit presents a steric hindrance in joining of 40S and 60S subunits. Only after complete maturation of 60S subunit, eIF6 is released from the 60S subunit so that the 40S subunit can join the 60S subunit to form a functional 80S ribosome (Gartmann *et al*., 2010).

Although it is known that adenovirus inhibit cap dependent translation during late times post infection identity of adenoviral protein involved in inhibiting cap dependent translation and exact mechanism is still not completely known (Huang & Schneider., 1991). Here, we report that BAdV-3 pVIII protein interacts with eIF6 and this interaction might prevent release of eIF6 from 60S subunit thereby preventing joining of 40S and 60S subunit resulting in impairment in the formation of functional 80S ribosome.

3.2 Materials and methods

3.2.1 Cell line and Viruses

Madin Darby Bovine Kidney (MDBK) cells (Kulshreshtha *et al*., 2004), cotton rat lung (CRL) cells (Papp *et al*., 1997) and VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) were grown in minimum essential medium (MEM; Sigma Aldrich) supplemented with 10% fetal bovine serum. Human embryo kidney (HEK) 293T cells (ATCC® CRL-3216™) were propagated in Dulbecco's Modified Eagle medium (DMEM)

supplemented with 10% FBS. BAV304a (contains BAdV-3 genome from which E3 region is deleted and is replaced by EYFP gene which is under CMV promoter) (Du & Tikoo., 2010) and mutant BAdV-3 were propagated in MDBK cells in MEM supplemented with 2% fetal bovine serum.

3.2.2 Antibodies

Polyclonal anti-pVIII serum (Ayalew *et al*., 2014), BAdV-3 DNA binding protein (DBP) (Zhou *et al*., 2001b), BAdV-3 pVII (Anand *et al*., 2014), BAdV-3 protein V (Kulshreshtha & Tikoo., 2008) BAdV-3 hexon (Patel & Tikoo., 2006) and BAdV-3 100K (Makadiya *et al*., 2015) specific antibodies have been described. Monoclonal antibody (Mab) specific to eIF6 (BD transduction laboratories), Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen), anti-β-actin monoclonal antibody, MAb specific to HA (Sigma-Aldrich), IRDye800 Conjugated goat anti-mouse antibody (Rockland), Alexa Flour 546 goat anti-mouse IgG antibody (Molecular probes), Alexa Flour 488 goat anti-rabbit IgG antibody, AP-conjugated goat anti-mouse antibody and Alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) were purchased.

3.2.3 Plasmid construction

Plasmid GC.linker, plasmid GN.linker and Plasmid pcDNA.3HA has been describrd earlier (Ayalew *et al*., 2014). Construction of plasmids pEY.VIII, pGB.VIII, pGA.VIII, pGST.pVIII, pGN.VIII and pGC.DDX3 has been described earlier (Ayalew *et al*., 2014). Following plasmids were constructed using standard DNA manipulation techniques.

i. ***pc3HA-eIF6***- PCR was done using primers eIF6-Fwd and eIF6-Rev (Table 3.2.1) and plasmid pGAD-eIF6 DNA as a template to amplify a 762bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product.

The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pc3HA-eIF6.

ii. ***pDS-eIF6***- PCR was done using primers DS- eIF6-Fwd and DS- eIF6-Rev (Table 3.2. 1) and plasmid pGAD-eIF6 DNA as a template to amplify a 759bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pDsRed-Monomer-C1 creating plasmid pDS-eIF6.

iii. ***pGC-eIF6***- PCR was done using primers GC- eIF6-Fwd and GC- eIF6-Rev (Table 3.2.1) and plasmid pGAD-eIF6 DNA as a template to amplify a 767bp DNA fragment containing eIF6 gene. Restriction enzymes *XmaI* and *NotI* were used to digest the PCR product. The digested PCR product was ligated to *XmaI* and *NotI* digested plasmid GC-linker in frame with the C-terminus of GFP gene creating plasmid pGC-eIF6.

iv. ***pC-pVIII***- PCR was done using primers pVIII-Fwd and pVIII-Rev (Table 3.2.1), and plasmid pFBAV302 (Zakhartchouk *et al*., 1998b) to amplify a 672bp DNA fragment containing pVIII gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA3.1 (-) (Invitrogen) creating plasmid pC-pVIII.

v. ***pC-pVIII-tn1***- PCR was done using primers pVIII-d1Fwd and pVIII-d1Rev (Table 3.2.1), and plasmid pC-pVIII DNA as a template to amplify a 417bp DNA fragment containing pVIII gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-tn1.

vi. ***pC-pVIII-tn2***- PCR was done using primers pVIII-d2Fwd and pVIII-Rev (Table 3.2.1), and plasmid pC-pVIII DNA as a template to amplify a 279bp DNA fragment containing pVIII gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-tn2.

vii. ***pC-pVIII-d3***- First round of PCR was performed with primer pairs pVIII-Fwd- pVIII-d3R and pVIII-d3F-pVIII-Rev to generate 426 and 160 bp products, respectively using plasmid pC-pVIII as template. PCR products of first round were mixed and used as templates with primers pVIII-Fwd and pVIII-Rev to amplify 543bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-d3.

viii. ***pC-pVIII-d4***- PCR was performed using primers pVIII-Fwd and pVIII-d4R (Table 3.2.1), and plasmid pC-pVIII DNA as a template to amplify a 549bp DNA fragment containing pVIII gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-d4.

ix. ***pC-pVIII-d5*** - First round of PCR was performed with primer pairs pVIII-Fwd- pVIII-d5R and pVIII-d5F-pVIII-Rev to generate 425 and 242 bp products, respectively using plasmid pC-pVIII as template. PCR products of first round were mixed and used as templates with primers pVIII-Fwd and pVIII-Rev to amplify 627 bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested

PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-d5.

x. ***pC-pVIII-d6***- First round of PCR was performed with primer pairs pVIII-Fwd- pVIII-d6R and pVIII-d6F-pVIII-Rev to generate 470 and 158 bp products, respectively using plasmid pC-pVIII as template. PCR products of first round were mixed and used as templates with primers pVIII-Fwd and pVIII-Rev to amplify 588 bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pC-cmyc creating plasmid pC-pVIII-d6.

xi. ***pC-3HAeIF6-tn1***- PCR was performed using primers eIF6-d1F and eIF6-d1R (Table 3.2.1) and plasmid pC-3HAeIF6 DNA as a template to amplify a 414bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pc3HA-eIF6-tn1.

xii. ***pC-3HAeIF6-tn2***- PCR was performed using primers eIF6-d2F and eIF6-d2R (Table 3.2.1) and plasmid pC-3HAeIF6 DNA as a template to amplify a 432bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pc3HA-eIF6-tn2.

xiii. ***pC-eIF6-del3***- PCR was performed using primers eIF6-d3F and eIF6-d2R (Table 3.2.1) and plasmid pC-3HAeIF6 DNA as a template to amplify a 606bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR

product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del3.

xiv. ***pC-eIF6-del4***- First round of PCR was performed with primer pairs eIF6-Fwd – eIF6-d4R and eIF6-d4F-eIF6-Rev (Table 3.2.1) to generate 186 and 480 bp products, respectively using plasmid pC-3HAeIF6 as template. PCR products of first round were mixed and used as templates with primers eIF6-Fwd and eIF6-Rev to amplify 624 bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del4.

xv. ***pC-eIF6-del5***- First round of PCR was performed with primer pairs eIF6-Fwd – eIF6-d5R and eIF6-d5F-eIF6-Rev (Table 3.2.1) to generate 324 and 351bp products, respectively using plasmid pC-3HAeIF6 as template. PCR products of first round were mixed and used as templates with primers eIF6-Fwd and eIF6-Rev to amplify 633 bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del5.

xvi. ***pC-eIF6-del-6***- PCR was performed using primers eIF6-d6F and eIF6-d2R (Table 3.2.1) and plasmid pC-3HAeIF6 DNA as a template to amplify a 630bp DNA fragment containing eIF6 gene. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del6.

xvii. ***pC-eIF6-del7***- First round of PCR was performed with primer pairs eIF6-Fwd – eIF6-d7R and eIF6-d7F-eIF6-Rev (Table 3.2.1) to generate 164 and 575 bp products,

respectively using plasmid pC-3HAeIF6 as template. PCR products of first round were mixed and used as templates with primers eIF6-Fwd and eIF6-Rev to amplify 693 bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del7.

xviii. ***pC-eIF6-del8***- First round of PCR was performed with primer pairs eIF6-Fwd – eIF6-d8R and eIF6-d8F-eIF6-Rev (Table 3.2.1) to generate 230 and 479bp products, respectively using plasmid pC-3HAeIF6 as template. PCR products of first round were mixed and used as templates with primers eIF6-Fwd and eIF6-Rev to amplify 669bp product by PCR. Restriction enzymes *EcoRI* and *XhoI* were used to digest the PCR product. The digested PCR product was ligated to *EcoRI* and *XhoI* digested plasmid pcDNA.3HA creating plasmid pC-eIF6-del8.

xix. ***pMCS+pVIII***- Restriction enzymes *MfeI* and *SpeI* were used to isolate a 7361 bp DNA fragment of plasmid pUC304a+ (Du & Tikoo., 2010). The isolated DNA fragment was then ligated to *MfeI-NheI* digested plasmid pMCS (Thanbichler *et al*., 2007) creating plasmid pMCS+pVIII.

xx. ***pMCS-minus-pVIII***- First round of PCR was performed and a 453 bp PCR fragment was amplified using primer pairs (FWD –Reco-p8-del \ Rev –Reco-p8-del-OE; Table 3.2.1) and plasmid pMCS+pVIII DNA as a template. Similarly, a 672 bp PCR fragment was amplified using primers (FWD –Reco-p8-del - FWD –Reco-p8-del-OE; Table 3.2.1) and plasmid pMCS+pVIII DNA as a template. The two PCR products were mixed and used as DNA templates with primers (FWD –Reco-p8-del + FWD –Reco-p8-del; Table 3.2.1) to amplify 1022 bp product by PCR. Restriction enzymes *AscI* and *AfeI* were used

to digest the PCR product. The digested PCR product was then ligated to *AscI* and *AfeI* digested plasmid pMCS+pVIII creating plasmid pMCS-minus-pVIII.

xxi. ***pMCS-pVIIIdel-Kana***- A 1240bp fragment was isolated from plasmid pUC4K (Taylor & Rose., 1988) by digesting it with *SbfI* and ligated to *SbfI* digested plasmid pMCS-minus-pVIII creating plasmid pMCS-pVIIIdel-Kana.

xxii. ***pUC304a.dVIII*** **-** Restriction enzyme *XcmI* was used to isolate a 6127 bp DNA fragment of plasmid pMCS-pVIIIdel-Kana. The isolated 6127bp DNA fragment was recombined with plasmid pUC304a+ DNA in *E. coli* BJ5183 to generate plasmid pUC304-pVIII-deleted-Kana. Finally, plasmid pUC304-pVIII-deleted-Kana was digested with restriction enzyme *Sbf*I and large fragment was self-ligated to generate plasmid pUC304a.dVIII.

xxiii. ***pMCS-d147-174-*** using primer pairs (FWD –Reco-p8-del - p8-del9-OE-Rev; Table 3.2.1) and plasmid pMCS+pVIII DNA as a template to amplify a 883 bp DNA fragment. Similarly, PCR was performed using primers (Rev-Reco-p8-del - p8-del9-OE-Fwd) and plasmid pMCS+pVIII DNA as template to amplify a 793 bp DNA fragment. The two PCR products were mixed and used as templates with primers (FWD –Reco-p8-del + Rev-Reco-p8-del; Table 3.2.1) to amplify 1636 bp product by PCR. Restriction enzymes *AscI* and *AfeI* were used to digest the PCR product. The digested PCR product was then ligated to *AscI* and *AfeI* digested plasmid pMCS+pVIII creating plasmid pMCS-d147-174.

xxiv. ***pUC304a-pVIII-d147-174-*** Restriction enzyme *XcmI* was used to isolate a 5446 bp DNA fragment from plasmid pMCS-d147-174. The isolated DNA fragment was used with *SbfI* digested plasmid pUC304a+ DNA for homologous recombination in E. *coli* BJ5183 to generate plasmid pUC304a-pVIII-d147-174.

Table 3.2.1 List of primers

Primer Name	Primer sequence
eIF6-Fwd	ATTGGTGAATTCATGGCGGTCCGTGCGTCGTTC
eIF6-Rev	GCCAATCTCGAGTCAGGTGAGGCTGTCAGTGAG
DS-eIF6-Fwd	ATTCTCGAGTTATGGCGGTCCGTGCGTCGTTCGAG
DS- eIF6- Rev	GCCGAATTCTACAGGTGAGGCTGTCAGTGAGGGAATC
GC- eIF6-Fwd	AATATATACCCCGGGATGGCGGTCCGTGCGTCGTTC
GC- eIF6-Rev	ATTAACGCGGCCGCTCAGGTGAGGCTGTCAGTGAG
pVIII-Fwd	GGCGCGGAATTCATGAGCAAAGAAATTCCCACACC
pVIII-Rev	GTCCTCGAGTCAGCTATAACCGCTCACAGAG
pVIII-d1Fwd	GGCGCCGAATTCATGAGCAAAGAAATTCCCACAC
pVIII-d1Rev	TATCTCGAGGGCGGTGAGCGGGATAAGGGTTC
pVIII-d2Fwd	GCGACAGAATTCGCCTCCTGCTTAAGACCAGATG
pVIII-d3F	TCGAACCCTTATCCCGTTAACCAGGCAGTTTGTAGAGGAATTTGT
pVIII-d3R	AAATTCCTCTACAAACTGCCTGGTTAACGGGATAAGGGTTCGAGC
pVIII-d4R	ACTGACCTCGAGTCAGGATCCTATGCCCCCGTGGCGCGGTCTG
pVIII-d5F	GAACCCTTATCCCGCTCACCTCGCGTTCATCTTTCAACCC
pVIII-d5R	GGGTTGAAAGATGAACGCGAGGTGAGCGGGATAAGGGTTC
pVIII-d6F	TCTTTCAACTAGGAGGAGGCAGGCAGTTTGTAGAGGAATT
pVIII-d6R	AATTCCTCTACAAACTGCCTGCCTCCTCCTAGTTGAAAGA
eIF6-d1F	ACTTTGGAATTCATGGCGGTCCGTGCGTCGTTCGAG
eIF6-d1R	CGAAGTCTCGAGAGCCAGGATCTCTTCTGTTTC

eIF6-d2F	ACTGACGAATTCTGCAATGACTATGTGGCCTTAGTCC
eIF6-d2R	ATTCTCGAGTCAGGTGAGGCTGTCAGTGAG
eIF6-d3F	ACTGACGAATTCTCCATCGCCGGCTGCCGCATC
eIF6-d4F	ACCATCCCCGTAGTGCACGCTGAGGAACGGCTCTCAGCCCTG
eIF6-d4R	CAGGGCTGAGAGCCGTTCCTCAGCGTGCACTACGGGGATGGT
eIF6-d5F	AGCGTGCAGATCCGGAGGGTGAAAGTGGCTGACCAGGTGCTG
eIF6-d5R	CAGCACCTGGTCAGCCACTTTCACCCTCCGGATCTGCACGCT
FWD–Reco-p8-del	TGACCAAAACCGCCACTCAGAGCAAAAAGAGC
FWD–Reco-p8-del-OE	GACCCGCCTAAACTCTCAGCCCTGCAGGGATAAGCCCTAATAGTAATC
Rev–Reco-p8-del	CGACTGCAGAATTCGAAGCTTGAGCTCGAGATC
Rev–Reco-p8-del-OE	GATTACTATTAGGGCTTATCCCTGCAGGGCTGAGAGTTTAGGCGGGTC
p8-del9-OE-Fwd	TCTTTCAACTAGGAGGAGGCAGGCAGTTTGTAGAGGAATT
p8-del9-OE-Rev	AATTCCTCTACAAACTGCCTGCCTCCTCCTAGTTGAAAGA
Q adeno Fwd	CAGGTGCCAGTCAAGATTAC
Q adeno Rev	ATGGCCGACTGAGTCATAAG
Actin Fwd	CTAGGCACCAGGGCGTAATG
Actin Rev	CCACACGGAGCTCGTTGTAG

3.2.4 Yeast two-hybrid assay

Initially, the interaction between pVIII and eIF6 was detected using the Matchmaker two-hybrid system3 using *Saccharomyces cerevisiae* (Clonetech) as described (Kulshreshtha & Tikoo., 2008). The plasmid DNA from indicated plasmids was used to cotransform yeast AH109 cells (Clontech). The transformed cells were then plated on to selective dropout medium plates

as described in (Makadiya., 2013) and the plates were then incubated at 30°C in a humidified environment for a duration of 5-7 days and observed for growth and development of color.

3.2.5 GST-pull down assay

The *Escherichia coli* BL21(D3E) pLysS (Promega) cells were used for expression of the recombinant GST-fusion proteins as described (Zhou & Tikoo., 2001). Purification of Glutathione S-transferase (GST) and GST-pVIII fusion proteins has already been described (Ayalew., 2015). The Bradford assay was used for measuring concentrations of the proteins. GST binding buffer (Ayalew., 2015) was used to wash and prepare glutathione sepharose beads for use in GST-pull down assay. Radio-labeled eIF6 was synthesized *in-vitro* by using plasmid pC -3HA-eIF6 DNA (1 μg) in an *in-vitro* transcription and translation reaction. The *in-vitro* transcription and translation reaction was carried out using a TNT T7 Coupled Reticulocyte Lysate System (Promega) and 30μCi of [^{35}S]-methionine (Perkin Elmer). Equal amount of GST or GST-pVIII fusion protein was mixed with 15μl of *in vitro* synthesized eIF6 and 40 μl of glutathione sepharose beads and incubated at 4°C overnight on a nutator. After the incubation, GST binding buffer was used to wash the beads three times. The beads were then boiled in 40 μl of Laemmli's sample buffer for 5 minutes. A 12% SDS-PAGE was run to separate the proteins. After the run was over the proteins in the gel were fixed using fixative solution for 30min. The gel was then dried using a geldryer (Bio-Rad Model 583). Finally, the dried gel was exposed to a phosphor screen (Kodak) for 16 hrs and visualized using Molecular Imager FX and Quantity One software (Bio-Rad).

3.2.6 Co-immunoprecipitation assay

293T cells were co-transfected with 4μg of each indicated plasmid DNA. At 48 hrs post transfection the cells were lysed in NP-40 lysis buffer and indicated protein specific antibodies

were used for immunoprecipitation of proteins from cell lysates. Subsequently, the proteins were run on 12% SDS-PAGE, transferred to PVDF membrane and probed by Western blot using indicated protein specific antibodies as described (Kulshreshtha & Tikoo., 2008). Similarly monolayer of MDBK cells grown in one well of 6 well plate were infected with BAdV-3 at an MOI of 5. At 48 hrs post infection the cells were lysed and processed as described above.

3.2.7 Bimolecular fluorescence complementation assay (BiFC)

Yeast S288c cells (A kind gift from Dr. Troy Harkness, University of Saskatchewan) were co-transformed, using the lithium acetate method (Gietz *et al*., 1992, Schiestl & Gietz., 1989) with indicated plasmid DNAs. The cells were then plated on to a selective drop out medium without urease and histidine and incubated at 30°C in a humidified environment. After 3-4 days of incubation, single colonies were picked, spread and fixed on a slide, mounted on a mounting medium containing DAPI (Vectashield) and examined using Zeiss LSM 5 laser scanning con-focal microscope.

3.2.8 Generation of stable cell line expressing BAdV-3 pVIII

For generation of lentivirus 293T cells in one well of 6 well plate were transfected with three plasmids pTrip-pVIII, pXPAX2 and pMD2.G. After 48 hours of transfection medium was collected, centrifuged at 1500 rpm for 5 min and then supernatant was filtered using 0.45µm filter and stored at -80°C. For transduction, VIDO DT1 cells in one well of 6 well plate were first treated with 8µg/ ml of polybrene for 15 minutes and then were either mock transduced or transduced with 500 µl of lentivirus. After 24 hours of transduction the media was changed to selection media (MEM with 2% fetal bovine serum and 10 µg/ml of puromycin). The selection media was changed every 48 hours until puromycin resistant clones begin to appear. The expression of pVIII in selected clones was confirmed by Western blot.

3.2.9 Polysome profile assay

MDBK cells in 100 mm culture dishes were either mock infected or infected with BAdV-3 virus at MOI of 2. After 40 hrs post infection, cyclohexamide (final concentration 50 μg per ml) was added to the medium and the cells were again incubated at 37°C for 30 minutes. After incubation, cold PBS was used to wash the cells two times. The cells were then scraped with a cell scrapper and collected in 2 ml tubes. Cells were then lysed by addition of polysome extraction buffer and incubation on ice. After incubating the cells on ice for 10 minutes, the cells were spun for 10 seconds to pellet the nuclei. Supernatant was collected and OD was taken at 260 nm. Equal OD units of individual cell lysates were applied to separate ultracentrifuge tubes containing 5%- 50% linear sucrose gradients. These tubes were then centrifuged in an ultracentrifuge (Beckman) at 4°C at 36000 rpm for 2 hours 30 minutes. From each tube, fractions of approximately 550μl were collected manually. The OD was measured from each fraction at 260nm and plotted on a graph against the fraction number. From each fraction total RNA was extracted using Trizol LS reagent (Invitrogen) following manufacturer's instructions. The Agilent 2100 Bioanalyser instrument was then used to analyse the extracted RNA.

For polysome profile analysis of pVIII expressing cell line, 70-80% confluent VIDO GT1 (Constitutively expressing BAdV-3 pVIII) and VIDO DT1 (Du & Tikoo., 2010) cells in 100 mm culture were processed as described above. Proteins were also extracted from top 1 to 16 fractions. Immunoblotting analysis of gradient fractions was performed using antibodies against eIF6 and BAdV-3 pVIII. Whole cell lysate was used as control.

3.2.10 Western blotting

MDBK cells were infected with either wild-type or mutant BAdV-3 at an MOI of 1. After 24 hrs, cell lysate was collected and viral proteins were detected by Western blotting using

protein specific rabbit antisera. Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen) was used as secondary antibody. β-actin was used as loading control and was probed by Western blot using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) as primary antibody and IRDye800 Conjugated goat anti-mouse antibody (Rockland) as secondary antibody. Finally, viral protein expression was analyzed by using the Odyssey infrared imaging system.

3.2.11 Isolation of mutant BAdV-3

Monolayers of VIDO DT1 cells in six well plates were transfected with 4-6μg of individual plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). At 4hrs post-transfection, the medium was replaced with fresh MEM containing 2% FBS. The cells were observed daily for appearance of any cytopathic effect (CPE). The cells showing CPE were collected, freeze thawed three times and used to purify the virus.

3.2.12 CsCl gradient centrifugation

Monolayers of MDBK cells were infected with individual virus. When more than 80% cells showed CPE, the cells were collected, pelleted by centrifugation and re-suspended in 5ml medium. The cell lysate was freeze thawed 5 times and subjected to CsCl density gradient centrifugation at 35000 rpm for 1hr at 4°C. The band containing virus was collected and used for a second round of CsCl density gradient centrifugation at 35000 rpm for 16 hrs at 4°C. The virus was collected, dialyzed against three changes of dialysis buffer to remove traces of cesium chloride and stored in small aliquots at -80°C.

3.2.13 Virus growth kinetics

MDBK cells in 24-well plates were infected with either BAdV304a or BAdV-3-d147-174 at MOI of 1. Infected cells were then collected at different time points (0, 6, 12, 24, 36, 48 h) post-infection, freeze-thawed 3 times and virus in cell lysate was titrated by $TCID_{50}$ in MDBK cells as described (Kulshreshtha *et al*., 2004).

3.2.14 Viral genome replication

Viral genome replication was measured as described in (Wu *et al*., 2012) with minor modifications. Briefly, MDBK cells were infected with either BAdV-304a or BAdV-3-d147-174 virus and harvested at indicated time points. Cells were pelleted and washed one time with phosphate-buffered saline (PBS). The cell pellet was then resuspended in isotonic buffer. Cell lysates were incubated on ice for 10 minutes and nuclei were then pelleted by centrifugation at 2000 × g at 4°C for 5 min and then 200 µl PBS was added for resuspension. DNA was then extracted using a DNeasy blood and tissue kit (Qiagen) and following manufacturer's instruction. Finally, the DNA was resuspended in double distilled water and subjected to quantitative PCR using Bio-Rad real time PCR system. The two sets of primers used for quantitative PCR were primer pair Q adeno Fwd and Q adeno Rev and primer pair Actin Fwd and Actin Rev (Table 3.2.1). Genome replication was calculated as the value of viral genome copy number divided by actin copy number. For comparison, the value of each virus was normalized to that of BAV304a at 6hpi and shown as relative viral genome copy number.

3.2.15 Transmission electron microscopy

The 300 mesh grids (Ted Pella, cat# G300) covered with 0.25% formvar and coated with carbon (Denton Vacuum Inc.) were suspended for 2 min on to a twenty microliter droplet of virus particles purified using CsCl gradient and fixed with 5% electron microscopy grade

glutaraldehyde (catalogue number 16316-10; Electron Microscopy Sciences). After 3 washes with water, the grids were suspended on a droplet of 0.5% Phosphotungstic Acid (J.B. Em Services Inc. Dorval QC) for 1 minute. The excess solution was removed and grids are allowed to dry. The grids with negatively stained virus particles were examined on an HT7700 transmission electron microscope (Hitachi Inc.) at 80 kv.

Monolayer of MDBK cells were infected with either BAdV-304a or BAdV-3-d147-174. At 48 hrs post-infection, the cells were harvested and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. Following fixation cells were pelleted in 1% Low melting point agarose, post fixed at room temperature in 1% OsO_4 in 0.1M sodium cacodylate buffer and en-bloc stained with saturated Uranyl Acetate in 70% Ethanol. Dehydration was carried out with a graded ethanol series and finalized in propylene oxide (Electron Microscopy Sciences cat#20401). Dehydrated cells were infiltrated with Epon812/Araldite embedding medium by gradually increasing concentration in propylene oxide, and then polymerized in molds with pure Epon/araldite at 60°C for 24 hrs. Finally, the pellet was sectioned with a Reichert ultracut ultramicrotome, each section stained with Reynolds lead citrate and 2% Uranyl acetate and viewed on a HT7700 transmission electron microscope (Hitachi Inc.) at 80 kv.

3.2.16 Infectivity assay

MDBK cells grown in 12 well plates were infected with equal number of virus ($TCID_{50}$). After 18 hrs 5 random fields were chosen from each well and GFP positive cells were counted for each well using fluorescent microscope. The average number of GFP positive cells were quantified as fluorescent focus unit (FFU) per field.

3.2.17 Statistical analysis

All data were analyzed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences among the groups were calculated using unpaired t-test. Differences were considered significant at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

3.3 Results

3.3.1 Interaction of BAdV-3 pVIII with cellular protein eIF6 *in-vitro*

Initially, results of yeast two-hybrid screen carried out using PCR amplified gene coding for BAdV-3 pVIII cloned into plasmid vector pGBKT7 in frame with GAL4 DNA binding domain as bait and plasmid vector pGADT7 (clontech) containing bovine retina cDNA library fused to GAL4 activation domain as prey, indicated that BAdV-3 pVIII interacts with cellular protein eIF6 (Patel and Tikoo unpublished results). To further confirm these findings, pVIII gene was cloned in frame to GAL4 activation domain (pGA.VIII) and used as prey. Similarly, eIF6 gene was cloned in frame to GAL4 DNA binding domain (pGB.eIF6) and used as bait. Yeast AH109 cells co-transformed using bait and prey vectors were screened for interaction as described earlier (Kulshreshtha & Tikoo., 2008). As seen in Fig.3.3.1 (panel A), yeast AH109 cells co-transformed with plasmid pGA.VIII and pGB.eIF6 DNAs revealed bluish green colonies suggestive of positive interactions. No such colonies were detected when AH108 cells were co-transformed with plasmid pGA.VIII and pGBKT7.

To confirm our initial yeast two-hybrid results, we performed the GST-pull down assay. Equal amount of purified GST or GST.pVIII fusion protein was individually mixed with sepharose beads and *in-vitro* transcribed and translated radiolabeled [^{35}S] eIF6 in an eppendorf tube and incubated at 4°C on a nutator. After washing the beads three times with washing buffer, the bound proteins were separated on 12% SDS-PAGE and analysed using Molecular Imager FX

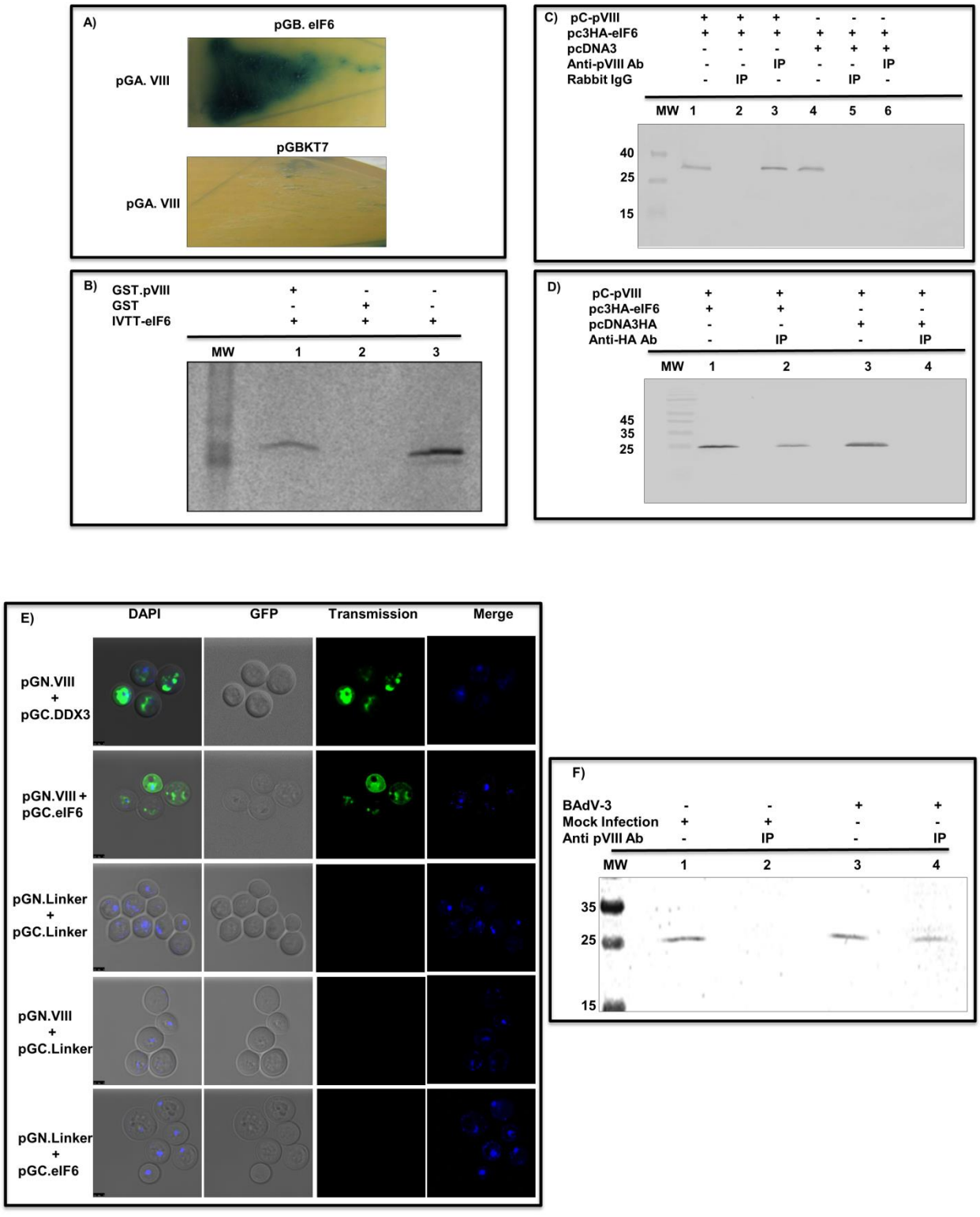


Figure 3.3.1 Interaction of BAdV-3 pVIII with eIF6. (A). Yeast two hybrid analysis. Plasmid DNA from pGA.VIII and pGB.eIF6 or pGA.VIII and pGBKT7 was used to co-

transform the AH109 yeast cells. The transformed yeast cells were streaked on a selective medium containing X-α-gal but devoid of Leu, Trp, Ade and His **(B). GST pull-down assay.** *In-vitro* translated [35S] methionine labeled HA tagged eIF6 was incubated with equal amount of either purified GST or GST.pVIII fusion protein immobilized on Glutathione-Sepharose 4B beads. The beads were washed three times and bead bound proteins were separated by 12% SDS-PAGE and detected by autoradiography. **(C). Co-immunoprecipitation assay in transfected cells.** Cell lysate was prepared from 293T cells co-transfected with either plasmid pC-pVIII and pc3HA-eIF6 DNA or plasmid pCDNA3 and pc3HA-eIF6 DNA. Proteins were immunoprecipitated from prepared cell lysate using anti-pVIII serum or anti-Rabbit IgG, run on 12% SDS-PAGE, transferred to PVDF membrane and probed in Western blot using anti-HA MAb. **(D). Co-immunoprecipitation assay in transfected cells.** Cell lysates were prepared from 293T cells co-transfected with either plasmid pC-pVIII and pc3HA-eIF6 DNA or plasmid pC-pVIII and pCDNA3HA DNA. Proteins were immunoprecipitated from prepared cell lysates using anti-HA MAb, run on 12% SDS-PAGE and transferred to PVDF membrane and probed in Western blot using anti-pVIII serum. **(E). Bimolecular fluorescence complementation.** Yeast S288c cells were co-transformed with indicated plasmids, plated on to a selective drop out medium and analyzed using Zeiss LSM 5 laser scanning confocal microscope as described in materials and methods. **(F). Co-immunoprecipitation assay in BAdV-3 infected cells.** Cell lysates were prepared from mock infected and BAdV-3 infected cells. Proteins from the prepared lysates were immunoprecipitated with anti-pVIII serum, run on 12% SDS-PAGE, transferred to PVDF membrane and probed in Western blot using anti-eIF6 MAb. Molecular weight markers (MW) are shown on the left of the panel.

and Quantity One software (Bio-Rad). As seen in Fig.3.3.1 (panel B), the radiolabeled eIF6 protein interacted with GST.pVIII fusion protein (lane 1) but not with GST protein alone (lane 2).

Next we validated these interactions in plasmid DNA transfected 293T cells *by* co-immunoprecipitation coupled with Western blot using protein specific antibodies. 293T cells were co-transfected with 4μg of each indicated plasmid DNA. At 48 hrs post transfection the cells were lysed and immuno-precipitated with indicated protein specific antibodies. Subsequently, the proteins were run on 12% SDS-PAGE, transferred to PVDF membrane and probed by Western blot using indicated protein specific antibodies. As seen in Fig.3.3.1 (panel C), eIF6 specific protein could be detected when proteins from the lysates of cells co-transfected with plasmid pC-pVIII + pc3HA-eIF6 DNAs were immuno-precipitated with anti-pVIII serum and probed in Western blot with anti-HA MAb (lane 3). No such protein could be detected when proteins from the lysates of cells co-transfected with plasmid pC-pVIII + pc3HA-eIF6 DNAs were immuno-precipitated with normal rabbit IgG and probed in Western blot with anti-HA MAb (lane 2). Similarly, no protein could be detected when proteins from the lysates of the cells co-transfected with plasmid pc3HA-eIF6 DNAs + pcDNA3 DNAs were immuno-precipitated with normal rabbit IgG (lane 5) or anti-pVIII sera (lane 6) and probed in Western blot with anti-HA Mab.

Similarly, pVIII specific protein (Fig.3.3.1. panel D) could be detected when proteins from the lysates of the cells co-transfected with plasmid pC-pVIII + pc3HA-eIF6 DNAs were immuno-precipitated with anti-HA MAb and probed in Western blot with anti-pVIII serum (Lane 2). No such protein could be detected in lysates of the cells co-transfected with plasmid pc3HA-eIF6 DNAs + pcDNA3 DNAs immuno-precipitated with anti—HA serum (lane 4).

3.3.2 Interaction of pVIII with eIF6 in cultured cells

Initially, we performed bimolecular fluorescence complementation assay. Indicated plasmid DNAs were used to co-transform yeast S288c cells. The transformed cells were plated on to a selective drop out medium without urease and histidine and incubated at 30^{o}C in a humidified environment. After 3-4 days of incubation, single colonies were picked, spread and fixed on a slide, mounted on a mounting medium containing DAPI and examined using con-focal microscope. As expected green fluorescence could be detected in yeast cells co-transformed with plasmid pGC.DDX3 + pGN.VIII DNAs (Ayalew., 2015). Similarly, green fluorescence could also be detected in yeast cells co-transformed with plasmid pGC.eIF6 + pGN.VIII DNAs (Fig.3.3.1. panel E). However, no green fluorescence could be detected in yeast cells co-transformed with plasmid pGN.VIII + pGC.linker, pGC.eIF6 + pGN.linker or pGN.linker + pGC.linker DNAs.

Finally, the interaction of viral pVIII with cellular eIF6 was verified in BAdV-3 infected cells. Monolayer of MDBK cells grown in one well of 6 well plates were infected with BAdV-3. At 48 hrs post infection, the cells were lysed, immuno-precipitated with anti-pVIII serum and probed in Western blot with anti -eIF6 MAb. As seen in Fig.3.3.1 (panel F), eIF6 protein could be detected in Western blots of mock infected (lane 1) or BAdV-3 infected (lane 3) cells using anti-eIF6 MAb. Similarly, eIF6 protein could be detected in the lysates of MDBK cells immuno-precipitated with anti-pVIII serum and probed in Western blot with anti eIF6 MAb (lane 4). No such protein could be detected in mock infected cells immuno-precipitated with anti-pVIII serum and probed in Western blot with anti-eIF6 MAb (Lane 2).

3.3.3 BAdV-3 pVIII domain interacting with eIF6

To further delineate the region of pVIII interacting with eIF6, we constructed a panel of plasmids expressing mutant pVIII proteins. Western blot analysis confirmed the expression of mutant proteins in transfected cells (Fig. 3.3.2.). Next, 293T cells were co-transfected with plasmid pc3HA-eIF6 and individual plasmid expressing mutant pVIII protein and analyzed by co-immunoprecipitation /Western blot analysis using protein specific antibodies. As seen in Fig.3.3.3. anti-pVIII serum co-immunoprecipitated eIF6 from the cells co-transfected with plasmid pc3HA-eIF6 + pC-pVIII-tn2 (panel B, lane 5) DNAs. Similarly, anti-pVIII serum co-immunoprecipitated eIF6 from the cells co-transfected with plasmid pC.pVIIId4+ pc3HA-eIF6 (panel C, lane 4) and plasmid pC-pVIII-d5 + pc3HA-eIF6 (panel D, lane 2) which could be detected in Western blot using anti-HA MAb. No eIF6 specific protein could be co-immunoprecipitated with anti-pVIII sera from the cells co-transfected with plasmid pc-3HA-eIF6 + pC-VIII-tn1 (panel B, lane 4), pC-pVIII-d3 + pc3HA-eIF6 (panel C, lane 2), pC-pVIII-d6 + pc3HA-eIF6 (panel C, lane 4). Detection of eIF6 (panel B, lane 3; panel C, lane 1 and lane3, panel D, lanes 1, lane 3, lane 5) represents input (10 %) protein.

3.3.4 eIF6 domain interacting with BAdV-3 pVIII

To identify the domain of eIF6 interacting with BAdV-3 pVIII, we constructed a panel of plasmids expressing mutant eIF6. Western blot analysis confirmed the expression of mutant proteins in transfected cells (Fig. 3.3.4.). Next, 293T cells were co-transfected with plasmid pC-pVIII and individual plasmid expressing mutant eIF6 proteins and analyzed by co-immunoprecipitation /Western blot analysis using protein specific antibodies. As seen in Fig.3.3.5 (panel B-D), anti-pVIII sera co-immunoprecipitated eIF6 mutant protein from the cells

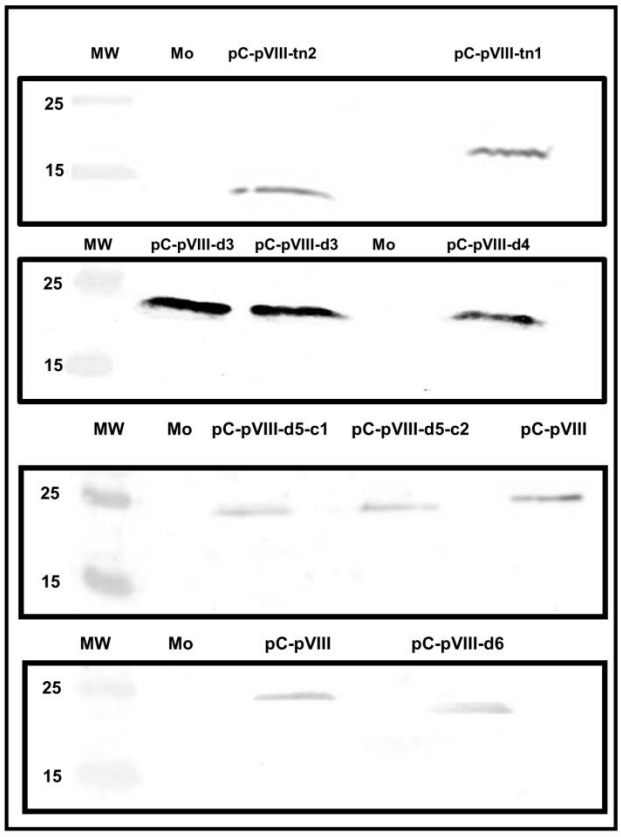


Figure 3.3.2 Expression of mutant proteins in transfected cells. Cell lysates were prepared from 293T cells transfected with indicated plasmid DNAs. Proteins from prepared lysates were run on 12% SDS-PAGE, transferred to PVDF membrane and probed using anti-pVIII serum. Molecular weight marker (MW). Mock transfected (Mo).

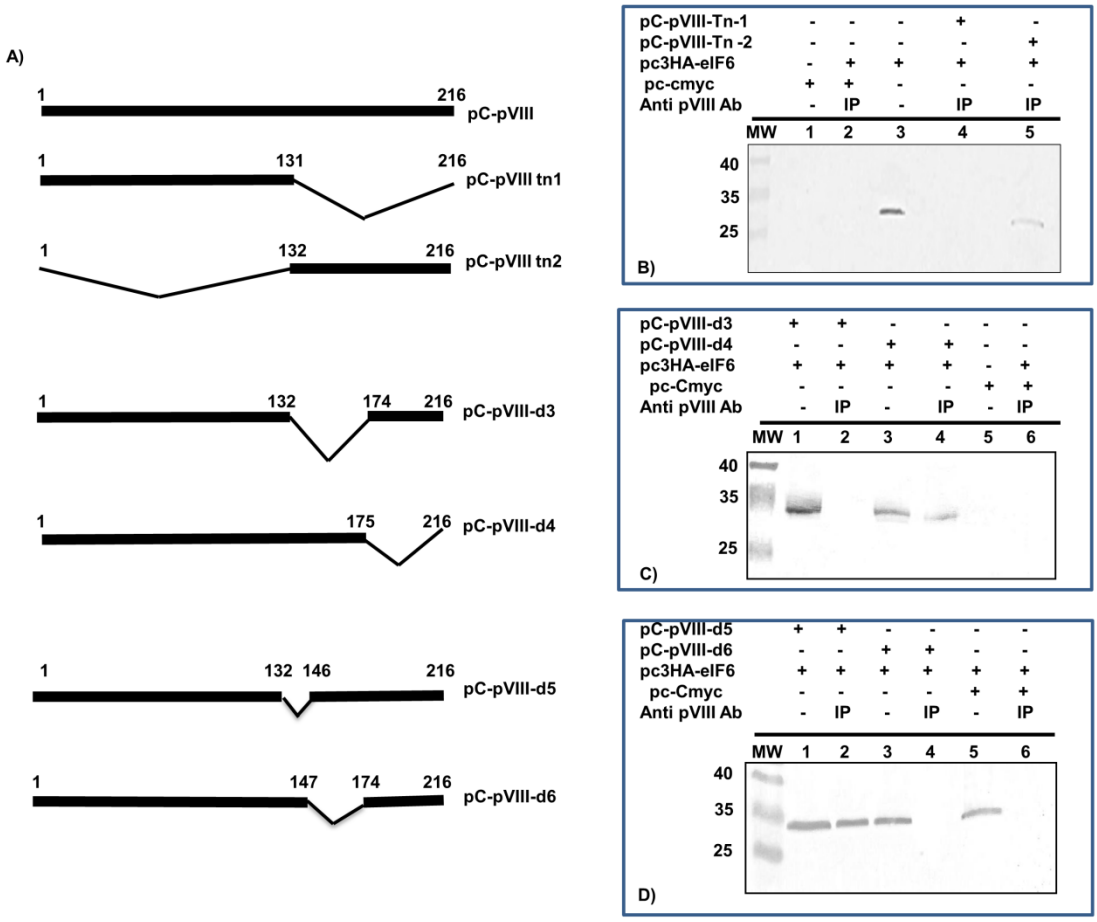


Figure 3.3.3 Identification of BAdV-3 pVIII domain interacting with eIF6. **(A) Schematic diagram of plasmids.** Thick line represents BAdV-3 pVIII gene. Thin lines represent deleted regions. The amino acid numbers of pVIII are indicated on the top. The name of the plasmid is depicted on the right of the panel. **(B-D) Western blot.** Cell lysates were prepared from 293T cells transfected with indicated plasmid DNAs. Proteins from the prepared cell lysates were immunoprecipitated with anti-pVIII serum (Ayalew *et al*., 2014), run on 12 % SDS-PAGE transferred to PVDF and probed using anti-HA MAb **(B-D).** Immunoprecipitation (IP), Molecular weight makers (MW).

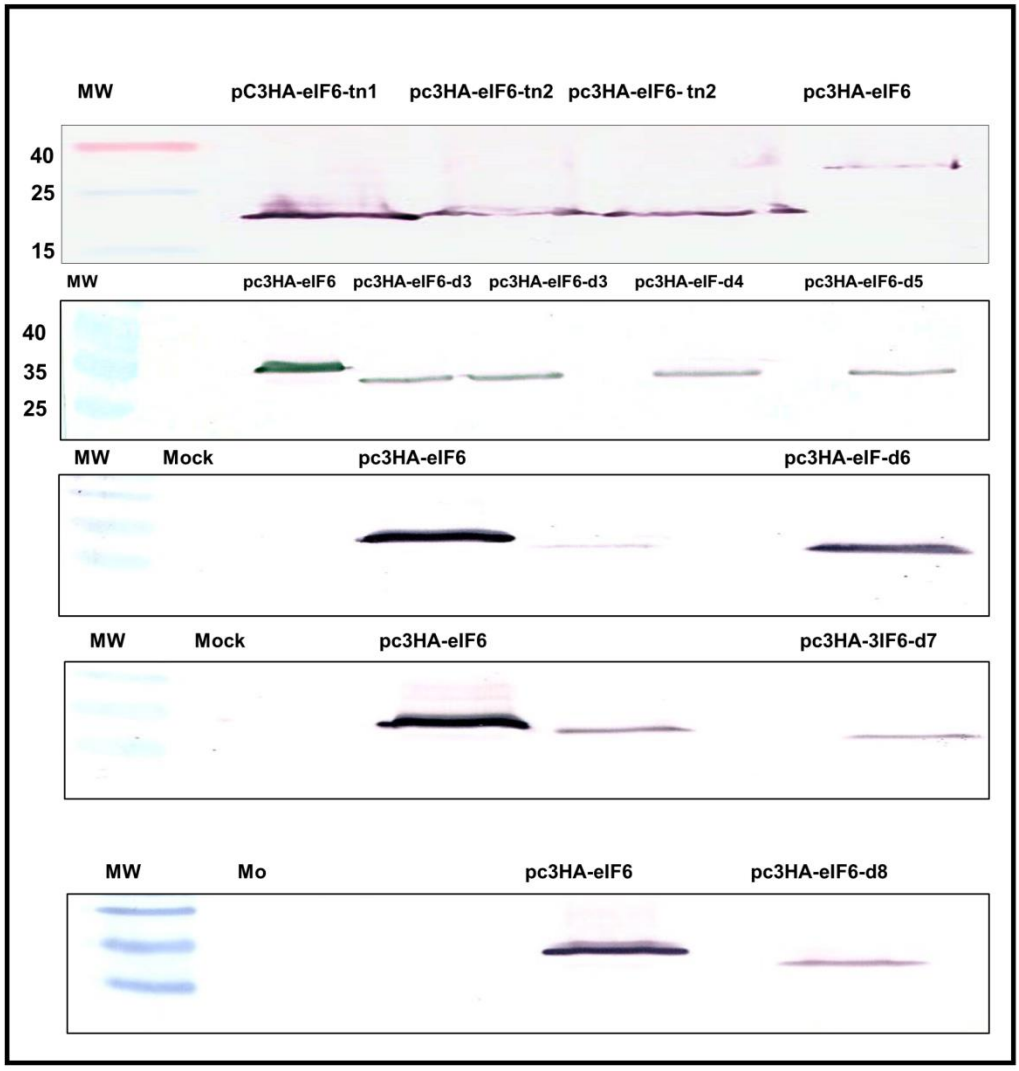


Figure 3.3.4 Expression of mutant proteins in transfected cells. Cell lysates were prepared from 293T cells transfected with indicated plasmid DNAs. Proteins from the prepared lysates were run on 12 % SDS-PAGE, transferred to PVDF membrane and probed using anti-HA Mab. Molecular weight marker (MW). Mock transfected (Mo).

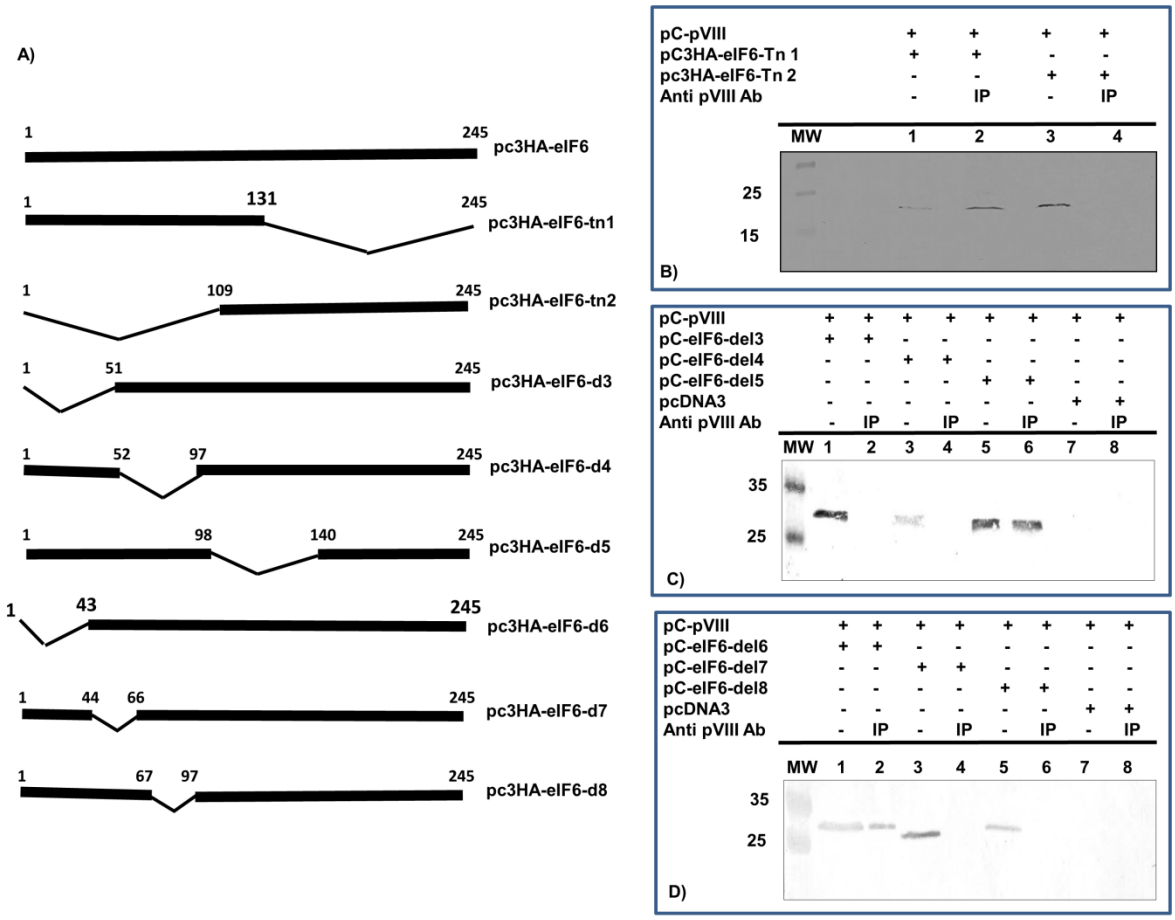


Figure 3.3.5 Identification of eIF6 region interacting with BAdV-3 pVIII. **(A). Schematic representation of plasmids.** Thick line represents eIF6 gene. Thin lines represent deleted regions. The amino acid numbers of eIF6 are depicted on the top. The name of the plasmid is depicted on the right of the panel. **(B-D). Western blot.** Cell lysates were prepared from 293T cells co-transfected with indicated plasmid DNAs. Proteins from prepared cell lysates were immunoprecipitated with anti-pVIII serum, run on 12% SDS-PAGE, transferred to PVDF membrane and probed using anti-HA MAb (B-D). Immunoprecipitation (IP), Molecular weight makers (MW).

co-transfected with plasmid pC.pVIII + pC.3HA-eIF6-tn1 (panel B, lane 2), pC-pVIII + pC.3HA-eIF6-d5 (panel C, lane 6) and pC-pVIII + pC.3HA-eIF6-d6 (panel D, lane 2) DNAs. No such protein could be co-immunoprecipitated from cells co-transfected with plasmid pC.pVIII + pc3HA-eIF6-tn2 (panel B, lane 4), pC-pVIII + pC3HA-eIF6-d3 (panel C, lane 2), pC-pVIII + pC3HA-eIF6-d4 (panel C, lane4), pC-pVIII + pc3HA-eIF6-d7 (panel D, lane 4), pC-pVIII + pc3HA-eIF6-d8 (panel D, lane 6) or pC-pVIII +pcDNA3 (panel C lane 8, panel D lane 8) Detection of eIF6 (panel B, lanes 1,3; panel C and D, lanes 1,3,5) represent input (10%) protein.

3.3.5 Analysis of polysome profiles

To determine if polysome profile is altered in BAdV-3 infected cells, we performed polysome profiling. MDBK cells were mock infected or infected with BAdV-3 at a MOI of 2. After 40 hrs post infection, cycloheximide was added to the medium. After further incubating at 37°C for 30 min, the polysome extraction buffer was added to lyse the cells and extract polysomes. Equal OD units of individual cell lysates were applied to ultracentrifuge tubes containing 5%- 50% linear sucrose gradients. These tubes were then centrifuged in an ultracentrifuge. From each tube, fractions of 550µl were collected manually. Total RNA was extracted from each fraction and the efficient generation of gradient and polysome separation were confirmed by analyzing extracted RNA by Agilent 2100 Bioanalyser instrument. As seen in Fig. 3.3.6, only 5S RNA is visible in top fractions (lanes 1 - 3) followed by fractions where only 18S RNA was present (lanes 4-7), followed by fractions in which both 18S RNA and 28S RNA were visible (lanes 8-21). Fractions 8 to 11 represent 60S ribosomal subunit, fractions 12 to 15 represent 80S ribosome and fractions 16 to 21 contained polysomes thus confirming efficient

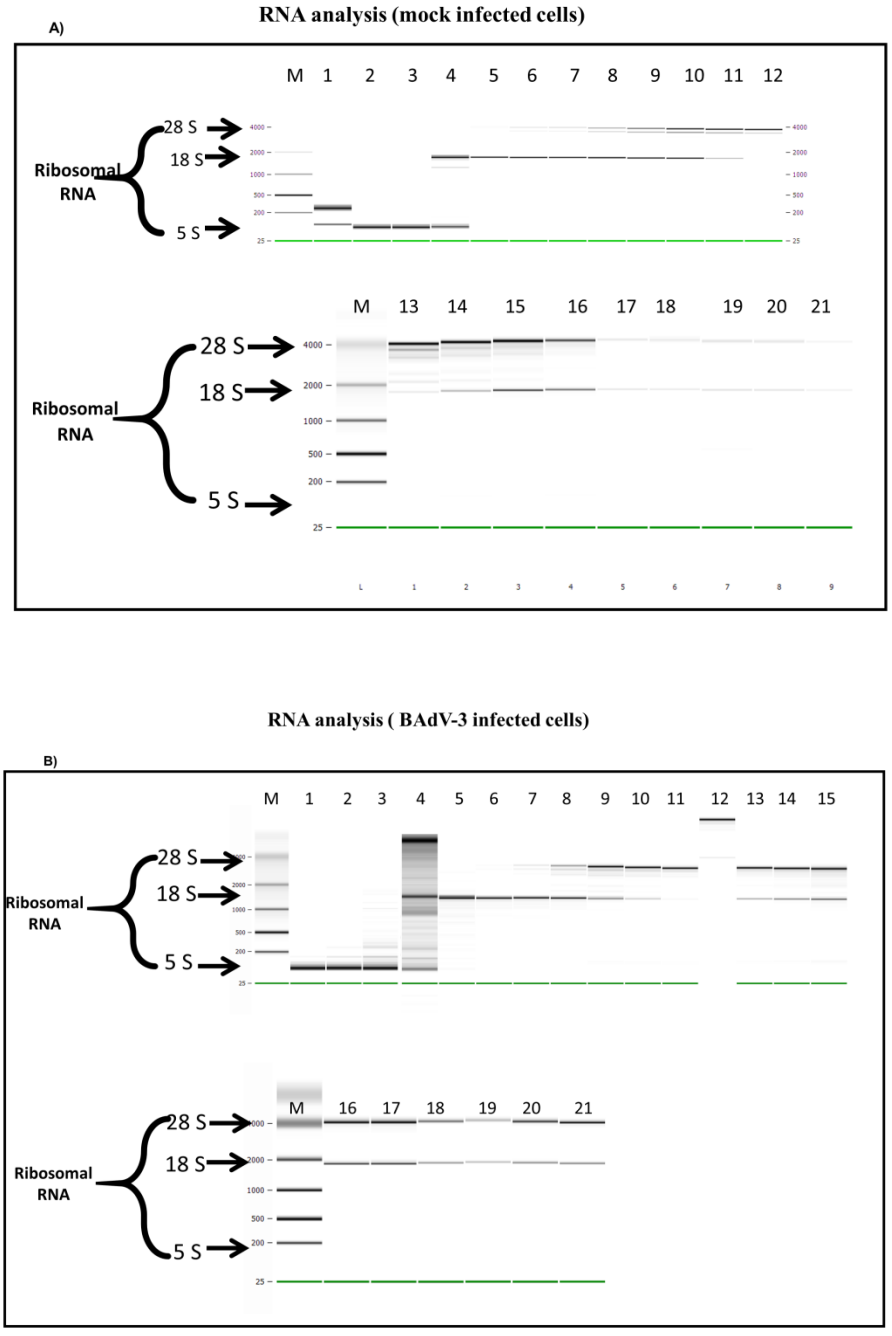


Figure 3.3.6 RNA analysis. Total RNA was extracted from each gradient fraction from **(A)** mock infected and **(B)** BAdV-3 infected MDBK cells using Trizol LS reagent (Invitrogen) and extracted RNA was by analyzed by Agilent 2100 Bioanalyser instrument. Fraction numbers (from top to bottom) are depicted on top.

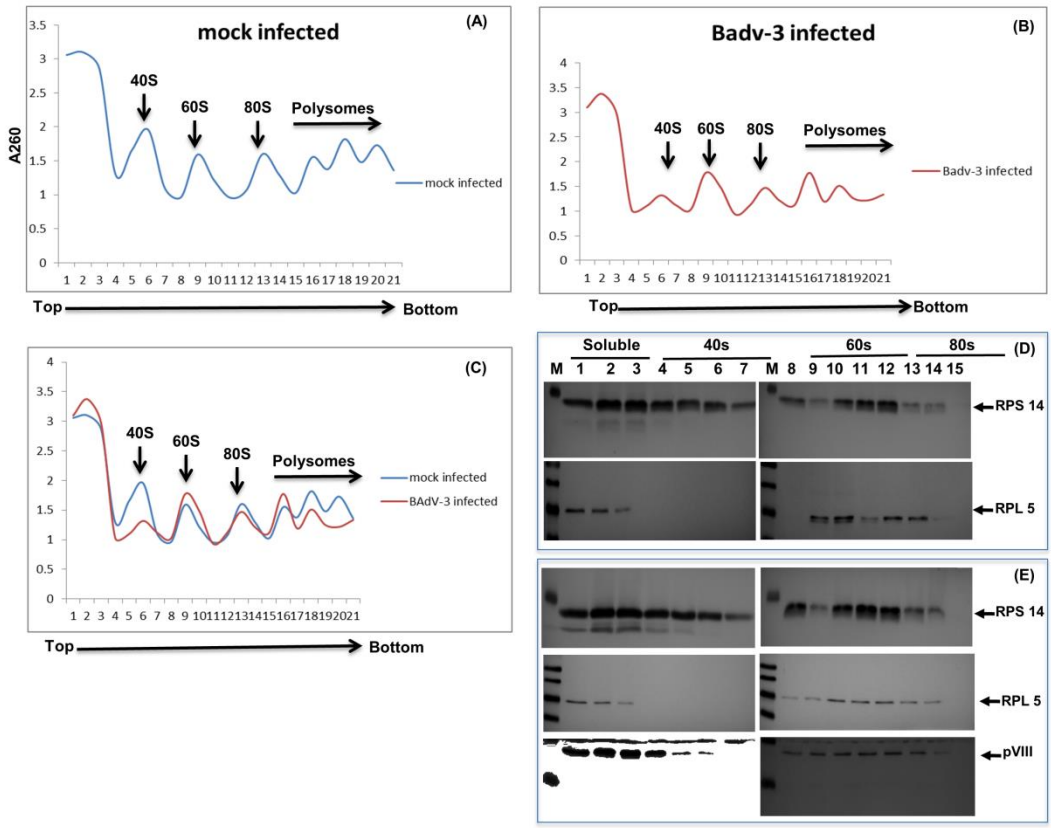


Figure 3.3.7 Polysome profile analysis. **(A-C). Polysome profile analysis of mock infected or BAdV-3 infected MDBK cells.** Cytoplasmic extracts were prepared from BAdV-3 infected or mock infected MDBK cells and subjected to 5 -50 % sucrose gradient centrifugation. Total RNA extracted from each gradient fraction was analysed by Agilent 2100 Bioanalyser instrument**.** The OD was measured at 260 nm for each fraction obtained from mock infected **(**panel A**)** or BAdV-3 infected cells **(**Panel B**)** and plotted on a graph against the fraction number. Polysomal profile of mock infected and BAdV-3 infected cells was compared **(**panel C). The monosomal (40S, 60S and 80S) and polysomal fractions are indicated. **(D-E). Western blot.** Proteins from the individual gradient fractions were run on 12% SDS-PAGE, transferred to PVDF and probed using protein specific antibodies. The number of the gradient fraction is depicted on the top of the panel. The name of the protein is depicted on the right of the panel.The data is representative of two independent experiments. Molecular weight marker (M).

generation of gradient. From each fraction OD was measured at 260nm and plotted on a graph against the fraction number (Fig.3.3.7. panels A-C). As seen in Fig.3.3.7 (panel C), compared to uninfected cells, polysomal peaks were smaller in infected cells. Moreover, while peak for free 60S was higher in infected cells, the free 40S peak was much smaller in infected cells. Proteins were extracted from each fraction and analysed by Western blotting using protein specific antisera. RPL5 (anti-RPL5 antibody) and RSP14 (anti-RPS14 antibody) proteins were used as marker for 60S ribosomes and 40S ribosomes, respectively. As seen in Fig 3.3.7, (panel D and panel E), RPL5 localized in the soluble fractions (Lane 1-3) and the fractions enriched in 60S ribosome (lanes 9-13 panel D and lanes 8-13 panel E), However, RPS 14 localized in the soluble fractions as well as in the fractions enriched in both 40S and 60S ribosome (Lanes 1-13 panel D and E) Similarly, pVIII localized in soluble fractions as well as fractions enriched in 40S ribosomes and 60S ribosomes (Lanes 1-13 panel E).

3.3.6 Isolation of lentivirus expressing pVIII

The VSV pseudotyped lentivirus expressing pVIII named TRIP.pVIII was isolated as described previously (Du & Tikoo., 2010). Briefly, 293T cells in 100 mm culture dishes were transfected with three plasmids pTrip-pVIII, pXPAX2 and pMD2.G. After 48 hours of transfection medium containing lentivirus was collected Generation of lentivirus was confirmed by using Lenti-X™ GoStix™ (Clonetech) following manufacturer's protocol.

3.3.7 Generation of stable cell line expressing BAdV-3 pVIII

To determine the effect of pVIII on polysome profile, we developed a stable cell line expressing BAdV-3 pVIII. Briefly, subconfluent VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) were transduced with lentivirus TRIP.pVIII. After 24 hrs of transduction, the media was changed to selection media (MEM with 2% fetal

bovine serum and 10 μg/ml of puromycin). The selection media was changed every 48 hours until puromycin resistant clones begin to appear. The puromycin resistant clones were expanded and analyzed by Western blotting. As seen in Fig.3.3.8, anti-pVIII sera detected pVIII specific protein in puromycin resistant cells (lanes 2 and 3) and BAdV-3 infected VIDO DT1 cells (lane 1). No such protein could be detected in VIDO DT1 cells (lane 4). The pVIII expressing cell line (clone1) designated as VIDO GT1 was used in the present study.

3.3.8 Ribosomal subunit association in pVIII expressing cells

To determine the effect of pVIII on polysomal profile, we compared the polysome profile of VIDO GT1 (expressing pVIII) and VIDO DT1 (not expressing pVIII) cells. As seen in Fig. 3.3.9 only 5S RNA is visible in lanes 1 to 3), 18S RNA is present in lanes 4 to 8, followed by fractions in which both 18S RNA and 28S RNA were visible, polysomes are present in fractions 14 to 21 thus confirming the efficient generation of the gradient. From each fraction OD was measured at 260nm and plotted on a graph against the fraction number (Fig.3.3.10, panels A-C). As seen in panel C, peaks for free 40S and free 60S were higher in VIDO GT1 (expressing pVIII) cells as compared to that in VIDO DT1 (not expressing pVIII) cells. In contrast, peaks of 80S and polyribosome were lower in VIDO GT1 cells as compared to VIDO DT1 cells.

Next we analysed proteins in the gradient fractions by Western blot using protein specific antibodies (Fig.3.3.10, panel D). As expected, anti-eIF6 MAb detected eIF6 in fractions enriched in 60S ribosome subunit of both VIDO DT1 and VIDO GT1 cells. However, anti-pVIII serum detected BAdV-3 pVIII protein in fractions enriched in 40S ribosome subunit as well as in fractions enriched in 60S ribosomal subunit of VIDO GT1 cells. In contrast, anti-pVIII serum did not detect BAdV-3 pVIII protein in polysomes of both VIDO DT1 and VIDO GT1 cells.

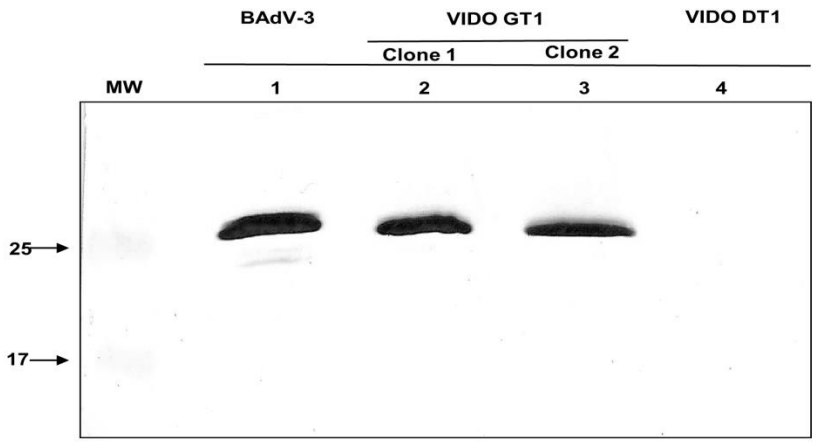
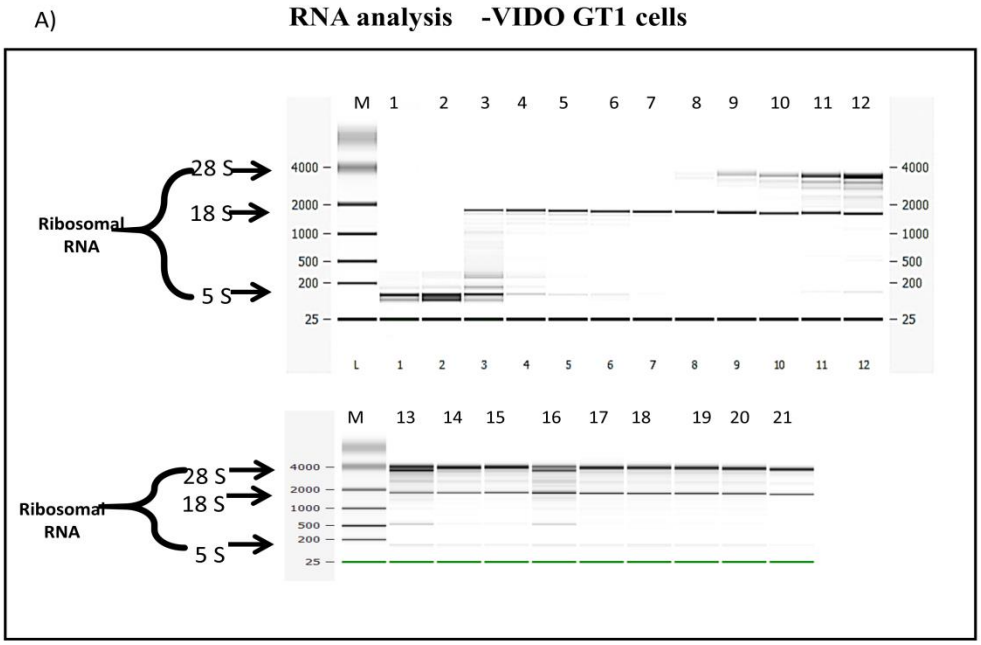


Figure 3.3.8 Analysis of pVIII expression in VIDO GT1 cells. Proteins from the cell lysates prepared from BAdV-3 infected VIDO DT1 cells (lane1), VIDO GT1 cells (lane 2 and 3) or VIDO DT1cells (lane 4) were run on 12 % SDS-PAGE, transferred to PVDF membrane and probed using anti-pVIII serum (Ayalew *et al*., 2014).



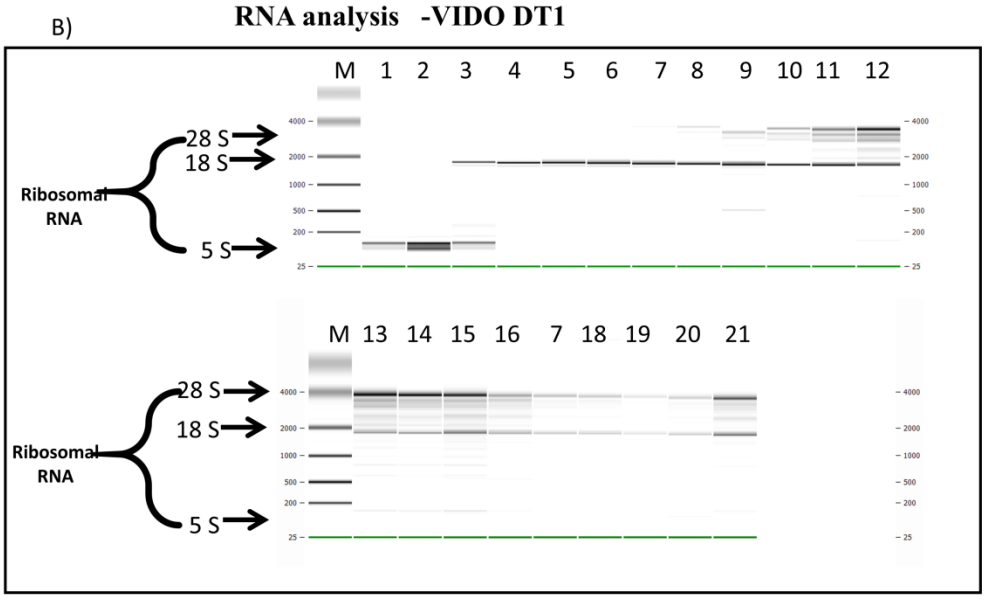


Figure 3.3.9 RNA analysis. Total RNA was extracted from each gradient fraction from **(A)** VIDO GT1 cells and **(B)** VIDO DT1 cells using Trizol LS reagent (Invitrogen) and extracted RNA was by analyzed by Agilent 2100 Bioanalyser instrument. Fraction numbers (from top to bottom) are depicted on top.

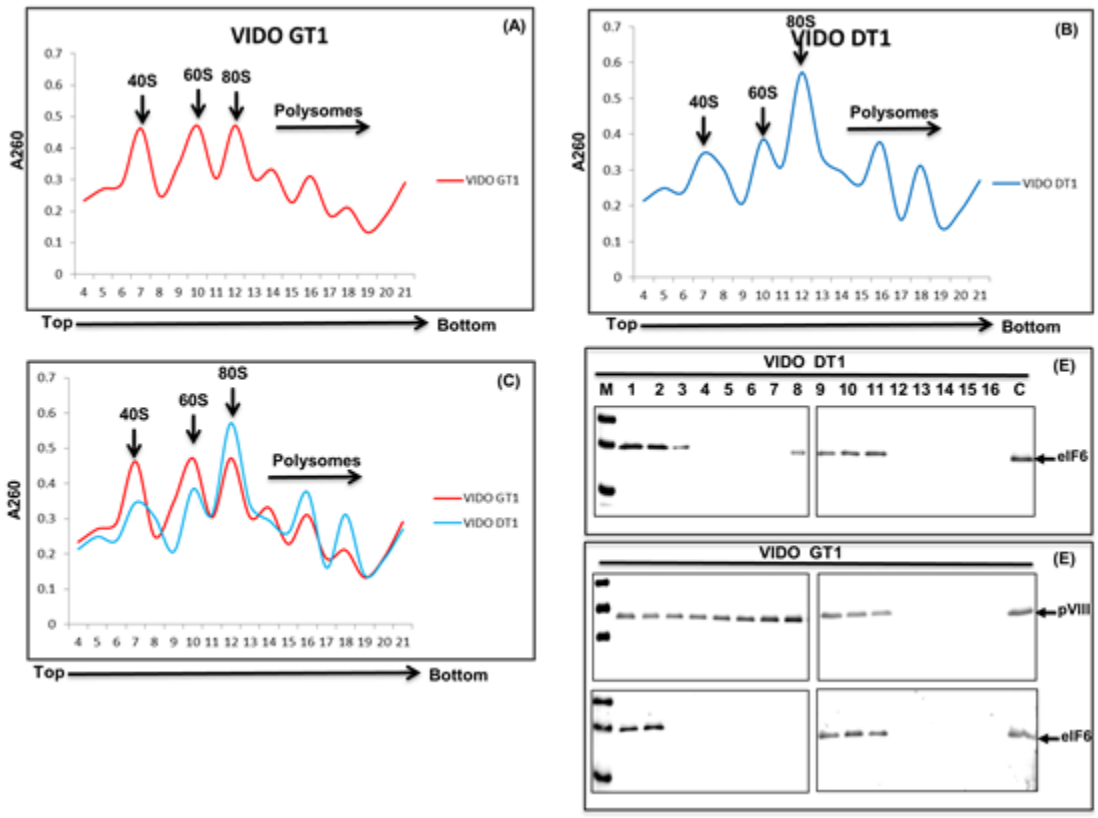


Figure 3.3.10 Polysome profile analysis. **(A-C). Polysome profile analysis of VIDO DT1 cells and VIDO GT1 cells (cells expressing BAdV-3 pVIII).** The cytoplasmic extract prepared from VIDO DT1 (Du & Tikoo., 2010) and VIDO GT1 (VIDO DT1 cells expressing BAdV-3 pVIII) cells was subjected to 5 -50 % sucrose gradient centrifugation. Total RNA extracted from each gradient fraction was analysed by Agilent 2100 Bioanalyser instrument. The OD 260 value was measured for each fraction obtained from VIDO GT1 (**A**) or VIDO DT1 (**B**) and plotted versus fraction numbers. Polysome profile of VIDO DT1 cells and VIDO GT1 cells was compared **(C). (D-E) Western blot.** Proteins from the individual gradient fractions were run on 12% SDS-PAGE, transferred to PVDF membrane and probed using protein specific antibodies. VIDODT1 (**D)**, VIDO GT1 (**E**). The number of the gradient fraction is depicted on the top of the panel. The name of the protein is depicted on the right of the panel. Molecular weight marker (M), Whole cell lysate from respective cells was used as control (C).

3.3.9 Construction of mutant BAdV-3

A plasmid pUC304a-pVIII-d147-174 containing full length BAV304a (contains BAdV-3 genome from which E3 region is deleted and is replaced by EYFP gene under CMV promoter) (Du & Tikoo., 2010) genomic DNA with deletion of the region encoding BAdV-3 pVIII amino acid 147 to 174 was constructed and used to transfect VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) The cells showing CPE and increased expression of GFP were harvested 25 day post transfection, freeze thawed three times and propagated in MDBK cells. The identity of mutant named BAVd147-174 was confirmed by sequencing of genomic DNA and by detection of recombinant protein in virus infected cells by Western blot using anti-pVIII sera (Fig.3.3.11A). As seen in Fig. 3.3.11A, anti-pVIII detected a 24 kDa protein in BAV304a infected cells (lane 1). In contrast, anti-pVIII serum detected a protein of 21 kDa in BAV.d147-174 infected cells (lane 2). No such protein could be detected in mock infected cells (lane 3).

3.3.10 Growth kinetics of BAdV-3-d147-174

To determine if the deletion of BAdV-3 pVIII domain interacting with eIF6 affects BAdV-3 replication in MDBK cells, we compared the growth kinetics of BAdV-304a and BAdV-3-d147-174. MDBK cells in 24-well plates were infected with either BAdV304a or BAdV-3-d147-174 at MOI of 1. At 0, 6, 12, 24, 36, 48 hrs post-infection, the cells were collected, freeze-thawed 3 times and virus in cell lysate was titrated by TCID50 in MDBK cells. As seen in Fig.3.3.11B, BAdV304a grew to a titer of $10^{8.5}$ $TCID_{50}$/ml while BAdV-3-d147-174 could only grow to a titer of 10^{6} $TCID_{50}$/ ml.

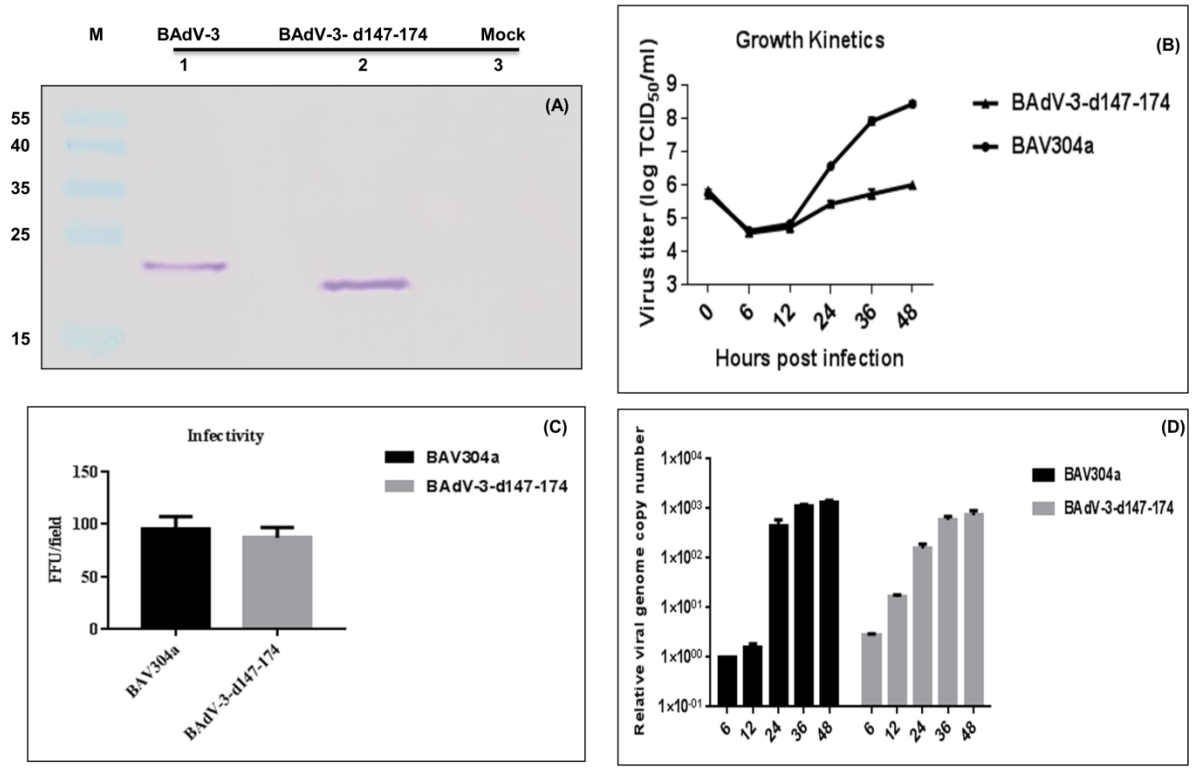


Figure 3.3.11 Characterization of mutant BAdV-3. **(A). Western blot.** Proteins from the lysates of BAdV-3 infected MDBK cells (lane 1), BAdV-3 d147-174 infected MDBK cells (lane 2) or mock infected MDBK cells (lane 3) were separated by 4-20% SDS-PAGE, transferred to PVDF membrane and probed using anti-pVIII serum (Ayalew *et al*., 2014). The size of the molecular Weight markers (M) in kDa are depicted on the left of the panel. **(B). Virus growth kinetics.** MDBK cells were infected with BAdV304a or BAdV-3-d147-174 at MOI of 1. At different times post infection, the infected cells were collected, freeze-thawed 3 times and virus in cell lysate was titrated by $TCID_{50}$ in MDBK cells. Values represent averages from two independent experiments and error bars indicate the standard deviations. **(C). Virus infectivity.** The MDBK cells were infected with equivalent amounts of infectious particles of indicated virus. The infected cells were visualized for the expression of GFP at 18 hrs post infection by

fluorescent microscope TCS SP5 (Leica). **(D). Viral genome replication.** The MDBK cells were infected with equivalent amounts of infectious particles of indicated virus in triplicate. At indicated times post infection, the infected cells were collected, and genomic DNA was isolated. The viral genome copy number was determined by quantitative PCR and divided by actin copy number for normalization. For comparison the normalized genome copy number values for each virus at each time point were compared to that of wild-type virus at 6hpi.

3.3.11 Virus Infectivity and genome replication

To test the virus infectivity, MDBK cells were infected with equal amount of BAV304a or BAdV-3-d147-174 and FFU were quantified from 5 random fields in each well. As seen in Fig.3.3.11C, there was no significant difference in the average number of FFU per field between BAV304a and BAdV-3-d147-174.

Viral DNA replication was analysed in MDBK cells infected with either BAdV-304a or BAdV-3-d147-174 using quantitative real time PCR. As seen in Fig.3.3.11D, there is not much difference in genome copy number during 6-12 hrs post infection. However, at 24 -48 hrs post infection, the genome copy number showed marginal difference (2 fold) between BAdV-3-d147-174 and BAV304a infected cells.

3.3.12 Analysis of protein expression in BAdV-3-d147-174 infected cells

To analyze the expression of viral proteins, MDBK cells infected with BAdV-304a or BAdV-3-d147-174 were collected, lysed and the lysates were used to detect the viral proteins by Western blotting using protein specific rabbit antisera. As seen in Fig.3.3.12A, there is no significant decrease in the expression of DBP (early protein) in the cells infected with BAdV-3-d147-174. However, there is significant decrease in the expression of analyzed BAdV-3 late proteins particularly hexon, pV and pVII in the cells infected with BAdV-3-d147-174.

3.3.13 Analysis of protein incorporation in BAdV-3-d147-174 viral particles

To analyse the incorporation of viral protein in the progeny virions, proteins from the purified virions were separated by 4-20% SDS-PAGE, transferred to PVDF membrane and probed in Western blot using viral protein specific serum. As seen in Fig.3.3.12B, no significant difference in the incorporation of the viral proteins in purified BAV304a or BAdV-3-d147-174 could be observed. Inspite of our repeated events we could not detect pVIII by western blotting

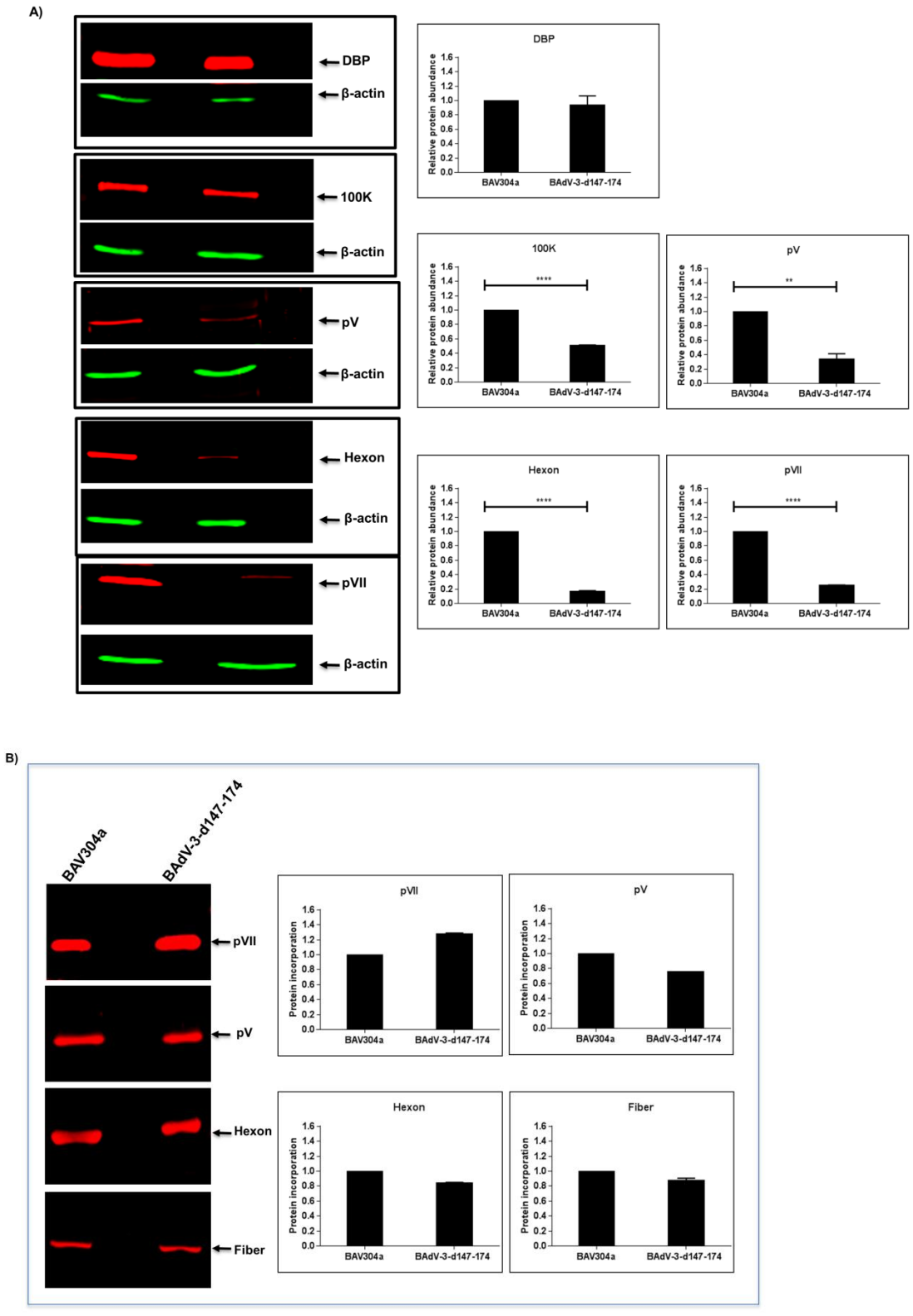


Figure 3.3.12 Analysis of viral proteins. **(A). Analysis of protein expression in infected cells**.

Proteins from the lysates of MDBK cells were separated by 4-20% gradient SDS-PAGE,

transferred to PVDF membranes and probed by Western blot using anti-DNA binding protein (DBP) (Zhou *et al*., 2001b), anti-100K (Makadiya *et al*., 2015) anti-pV (Kulshreshtha & Tikoo., 2008),anti- hexon (Patel & Tikoo., 2006) and pVII (Anand *et al*., 2014) sera followed by Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). β-actin was detected by Western blot using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by IRDye800 conjugated goat anti-mouse antibody (Rockland). The name of the proteins is depicted on the right of the panel. DBP (DNA binding protein). The results were analyzed by using Odyssey Infrared Imaging System and are presented as bar diagram. Values represent averages from two independent repeats and error bars indicate the standard deviations. **(B). Analysis of viral protein incorporation in purified virions.** Proteins from purified BAdV304a, or BAdV-3-d147-174 were separated by 4-20% gradient SDS-PAGE, transferred to PVDF membrane and probed in Western blot using protein specific antisera. The name of the detected protein is depicted on the right. The results were analyzed by using Odyssey Infrared Imaging System and are presented as bar diagram. Values represent averages from two independent repeats and error bars indicate the standard deviations.

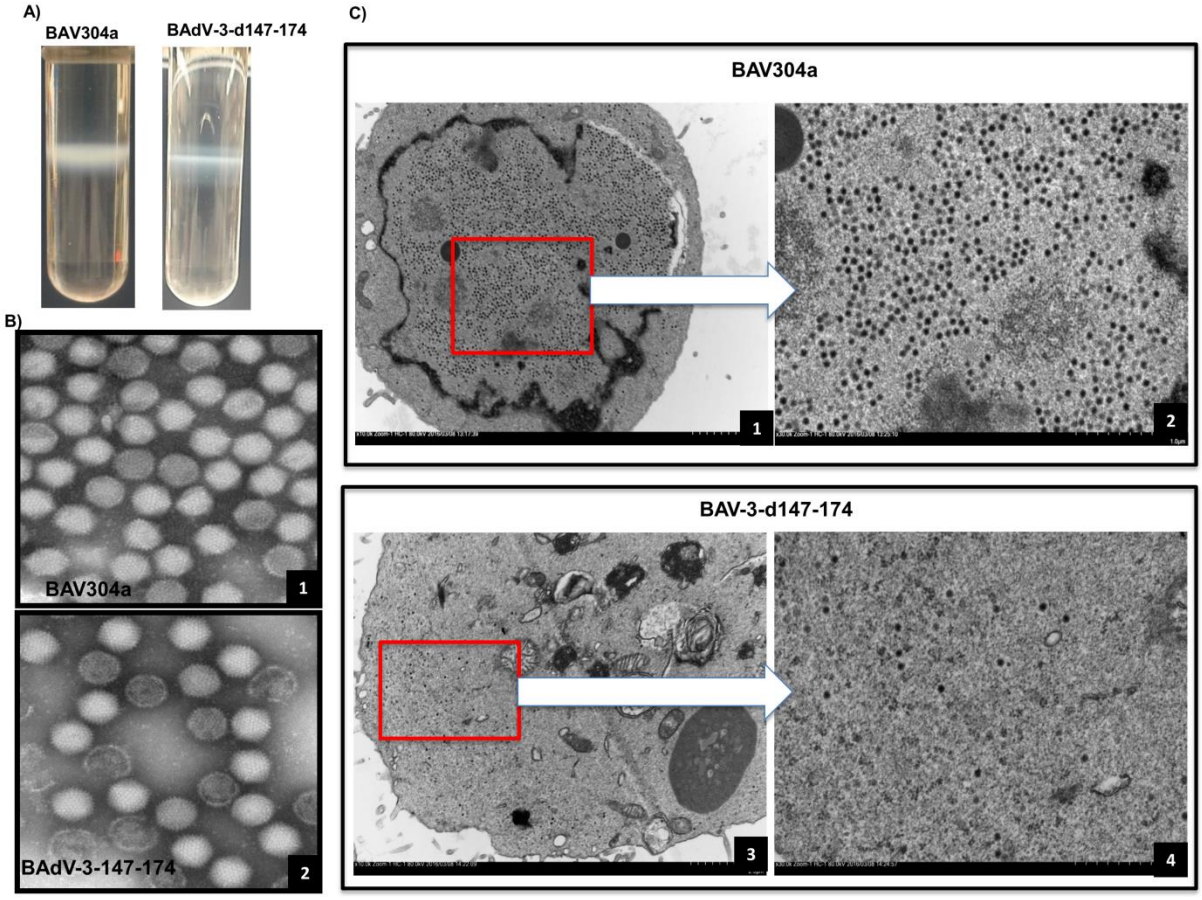


Figure 3.3.13 Analysis of BAdV-3-d147-174 assembly. **(A). CsCl2 purification.** Virus was purified from infected cell lysates by $CsCl_2$ density gradient purification. Mature virion bands after double density-gradient centrifugation. **(B). Electron microscopic analysis of purified virus.** Purified BAV304a (panel 1), BAdV-3-d147-174 (panel 2) (Magnification 100000×). **(C). Electron microscopic analysis of virus infected cells.** BAV304a infected (panel 1) or BAdV-3-d147-174 (panel 3) infected MDBK cells (Magnification 10000×). The arrows depict the enlargement of selected boxed region of panel 1 (panel2) and panel 3 (panel 4) (Magnification 30000×).

of purified BAdV-3-d147-174 this may be because the size of C-terminal cleaved fragment of pVIII is small and it might pass through the membrane during transfer from gel to membrane.

3.3.14 Analysis of BAV-3-d147-174 assembly

To assess whether deletion of amino acid 147-174 of pVIII has any effect on the virus structure, the BAV304a or BAV-3-d147-174 were propagated in MDBK cells and purified by CsCl density gradient as described (Tollefson *et al*., 2007). The number of MDBK cells used to purify BAV-3-d147-174 was three times more than that used for purifying BAV304a. As seen in Fig.3.3.13A, the amount of mature virions present in BAdV3-d147-174 infected cells appears lower than the amount detected in BAV304a infected cells. The negative-staining electron microscopy revealed that like BAV304a, the BAdV-3-d147-174 particles appeared icosahedral in shape (Fig.3.3.13B). However, unlike BAV304a, more BAV-3-d147-174 virus particles appeared disrupted (Fig.3.3.13B).

To further assess whether deletion of BAdV-3 pVIII amino acids numbered 147-174 has any effect on virus assembly, transmission electron microscopy was performed on MDBK cells infected with BAdV304a or BAdV-3-d147-174. As seen in Fig.3.3.13C, TEM images of virions suggested no observable defect in the assembly of virus particles. However, unlike BAV304a, few virus particles with darkly staining core were detected in BAV-3-d147-174 infected cells.

3.4 Discussion

Protein-protein interactions influence every cellular process. Since, the interaction of viral proteins with host cell proteins is essential for virus growth and survival, identifying the interactions and determining the consequence of such interactions is important in understanding the virus –host interactions (Phizicky & Fields., 1995). Earlier, we demonstrated that BAdV-3 pVIII is expressed as 24 kDa protein in BAdV-3 infected as well as BAdV-3 pVIII transfected

cells and is transported to nucleus by interacting preferentially with α3 importin (Ayalew *et al*., 2014). We also demonstrated that pVIII interacts with cellular factor DDX3 and inhibits cap dependent translation (Ayalew., 2015). Here, we report a new interaction between BAdV-3 pVIII and cellular protein eIF6, which appear to alter the formation of functional 80S ribosomes and may modulate cellular protein translation. Moreover, pVIII domain interacting with eIF6 appears to be important for efficient replication of BAdV-3.

The eukaryotic initiation factor 6 is a 245 amino acid extremely conserved protein (Si *et al*., 1997,Si & Maitra., 1999), which is important for ribosome biogenesis and protein translation (Miluzio *et al*., 2009). Several lines of evidence suggest that pVIII and eIF6 interact during BAdV-3 infection. First, yeast two hybrid experiments indicated that pVIII interacts with eIF6. Secondly, pVIII-eIF-6 interaction was demonstrated using GST pulled down assay. Thirdly, bimolecular fluorescence complementation assay suggested that pVIII and eIF6 interact *in- vivo*. Finally, using co-immunoprecipitation and Western blot assays, we demonstrated that cellular eIF6 interact with pVIII synthesized in BAdV-3 infected cells. Deletion analysis revealed that C-terminus of pVIII (amino acids 147-174) appear to interact with N-terminus of eIF6 (amino acids 44-97).

Earlier studies involving yeast cells reported that depletion of Tif6 (yeast eIF6 homologue) results in rapid reduction of 60S ribosomal subunits compared to 40S subunit. (Sanvito *et al*., 1999,Si & Maitra., 1999,Wood *et al*., 1999). Moreover, the eIF6 also specifically binds to free 60S ribosomal subunit and prevents premature association of pre-40S and pre-60S subunits in absence of mRNA joining with 40S ribosomal subunit (Gartmann *et al*., 2010,Russell & Spremulli., 1979,Valenzuela *et al*., 1982). For initiation of translation, eIF6 is released from the 60S subunit in the cytoplasm (Ceci *et al*., 2003), which allows joining of 60S and 40S subunits

resulting in the formation of active, translating 80S complex (Ceci *et al*., 2003,Horsey *et al*., 2004,Si & Maitra., 1999). Our results support the suggestion that interaction of eIF6 with pVIII alters the release of eIF6 from the 60S subunit in infected \ transfected cells, which lead to the reduction in the formation of functional 80S subunits. First, there are more free 60S ribosomal subunits and less 80S ribosomal subunits in BAdV-3 infected cells than mock infected cells. Second, there are more free 60S ribosomal subunits and less 80S ribosomal units in cells expressing BAdV-3 pVIII compared to control cells.

Earlier report demonstrated that reduction of 40S ribosomal subunit modulates the hepatitis C virus IRES-mediated translation but not host specific translation (Huang *et al*., 2012). Analysis of BAdV-3 infected cell polysomes revealed the reduction in 40S ribosomal subunits in infected cells. Although the role of such reduction in 40S ribosomal subunits in BAdV-3 infected cells is not yet clear, the absence of such reduction in 40S ribosomal subunits in pVIII expressing cell line suggests that pVIII is not involved in this process.

A number of viruses are known to target initiation factors and translation initiation step to inhibit host mRNA translation (Bushell & Sarnow., 2002,Schneider & Mohr., 2003). Some of the strategies used by viruses to target translation initiation and inhibit host protein synthesis includes, modification of activity of eukaryotic initiation factors (Feigenblum & Schneider., 1993), targeting PABP and sequestering it (Ilkow *et al*., 2008,Smith & Gray., 2010), cleavage of eIF4G and/ or de-phosphorylation of 4EBP1 (You *et al*., 1999), modulating activity of eIF4E (Burgui *et al*., 2007,Connor & Lyles., 2002) or excluding 4EBP1 from mRNA cap binding complex (Aoyagi *et al*., 2010) by direct interaction of viral proteins with eIFs.

The cellular mRNA translation is also inhibited in adenovirus infected cells at late times post infection (Huang & Schneider., 1991). Earlier report suggested that interaction of

adenovirus 100K protein with eIF4G and consequent displacement of mnk-1 from eIF4G leading to under-phosphorylation of eIF4E results in altering cellular mRNA translation (Cuesta *et al*., 2000,Cuesta *et al*., 2004). Recent report has suggested that the cellular mRNA translation may also be interfered by altering the availability of eIFs for cap binding protein complex due to interaction of pVIII with DDX3 (Ayalew., 2015). We speculate that this impairment of formation of functional 80S unit, because of inhibition of release of eIF6 from 60S subunit may be yet another mechanism of altering the translation of cellular mRNAs at late times post BAdV-3 infection and helping the preferential translation of late viral mRNAs by ribosome shunting mechanism (Yueh & Schneider., 1996). In ribosome shunting mechanism, the 40S ribosomal subunit binds to the cap structure and scans a short linear non structured region of mRNA. It then shunts all the structured region having secondary structure and hairpin loops and lands to a site several hundred nucleotides downstream. Since the structured region is not scanned the secondary structure of shunted region remains preserved (López-Lastra *et al*., 2010). All adenovirus late mRNAs have tripartite leader spliced to them. The tripartite leader which acts as a shunting element has a proximal linear non structured region which is followed by highly structured region which has complex secondary structures and hairpin loops (Yueh & Schneider., 1996,Yueh & Schneider., 2000). At late time points post adenovirus infection when host cap dependent protein synthesis is inhibited adenovirus late mRNAs are preferentially translated by ribosome shunting mechanism (Xi *et al*., 2004). Thus, it appears that inhibition of cellular mRNA translation at late times post infection may be mediated by multiple mechanisms involving different adenovirus late proteins. However, it is not clear if different mechanisms of inhibition of cellular mRNA translation in adenovirus infected cells at late times post infection act synergistically or act separately at different times post infection.

Domain of BAdV-3 pVIII (amino acid 147-174) interacting with eIF6 (amino acid 44 to 97) appears dispensable for the production of progeny virus. Although formation of mature BAdV-3-d147-174 virus occurs, the mutant virus yield and production of mature virus particles (Fig.3.3.11 panel B; Fig.3.3.13, panel A, B, C) appears affected. Both BAdV-3-d147-174 and BAV304a showed comparable infectivity and viral genome replication. Moreover, deletion of eIF6 interacting domain of pVIII (amino acid 147-174) does not affect the structure of the capsid of mature BAdV-3-d147-174 as it does not make it thermolabile. However, analysis of BAdV-3-d147-174 gene expression revealed that while the expression of DBP (early gene) is not affected, the expression of late viral proteins was significantly decreased in BAdV-3-d147-174 infected cells. However, there was no significant difference in the incorporation of analyzed structural proteins between BAdV-3-d147-174 and BAV304a. These results indicate that abrogating the interaction of pVIII and eIF6 affect the expression of adenovirus late proteins, which may lead to decrease in the production of mature virus particles. We suggest that absence of interaction of pVIII and eIF6 in BAdV-3-d147-174 allows the formation of 80S ribosomal subunit required for efficient translation of cellular mRNAs, thus modulating the preferential translation of adenoviral mRNAs during late phase of infection in BAV304a infected cells.

In conclusion, our results suggest that C- terminus (amino acid 147 to 174) of pVIII interact with N- terminus (amino acids 44 to 97) of eIF6, which causes impairment of the formation of 80S ribosomes and may lead to modulation of cellular mRNA translation at late times post BAdV-3 infection. We speculate that this impairment of ribosomal subunit joining is a consequence of inhibition of release of eIF6 from 60S subunit by pVIII. Moreover, we have demonstrated that abrogation of this interaction affects the formation of mature BAdV-3 virions and virus yield by modulating the translation of BAdV-3 mRNAs at late times post infection.

4.0 TRANSITION FROM SECTION 3.0 TO SECTION 5.0

Protein-protein interactions are integral part of virus life cycle. Viral proteins often interact with host cell proteins and other viral proteins to perform their functions. So far, I analysed the interaction of pVIII with one of the cellular protein eIF6 and determined the biological significance of this interaction. In the next section, I studied the interaction of pVIII with BAdV-3 protease. The following section describes characterization of adenovirus protease mediated cleavage of pVIII and the role of proteolytic cleavage of pVIII in virus assembly, stability and infection.

5.0 ROLE OF PROTEOLYTIC CLEAVAGE OF BOVINE ADENOVIRUS-3 ENCODED PVIII IN BAdV-3 INFECTION

5.1 Introduction

Adenoviruses are complex, large, non-enveloped viruses that have a linear double stranded DNA genome (Martin *et al*., 2007). The in-depth molecular characterization of human adenoviruses and their properties like ease of genome manipulation, broad tropism, transgene carrying capacity of up to 30kb and rapid growth to high titers in tissue culture have made adenoviruses a popular tool for gene transfer into mammalian cells (Campos & Barry., 2007,Tatsis & Ertl., 2004) . However, despite being extensively studied for more than 5 decades some aspects of adenovirus biology such as assembly and final maturation are not fully understood. Moreover, localization of some of minor capsid proteins and their role(s) in adenovirus lifecycle is not clear yet (Condezo *et al*., 2015).

Like many other viruses and bacteriophages, proteolytic maturation is a key step in the life cycle of adenoviruses. The key player in adenovirus maturation is adenovirus protease which recognizes and cleaves at two consensus cleavage motifs (M/I/L)XGX-G and(M/I/L)XGG-X (where X is any amino acid) (Webster *et al*., 1989). Adenoviral proteins that are substrate for adenovirus protease include three minor capsid proteins (pIIIa, pVI and pVIII) and three core proteins (pVII, pμ and pTP) (Challberg & Kelly., 1981,Mangel *et al*., 1996,Weber., 1976). Apart from these six structural proteins two nonstructural proteins L1 52/55k (Perez-Berna *et al*., 2014) and 100K (Makadiya *et al*., 2015) have been recently reported to be substrates of adenoviral protease.

One of the substrate for adenoviral protease is pVIII. The pVIII is a minor capsid protein that is present on the inner surface of the capsid and connects the core with the capsid (Rohn *et*

al., 1997). As pVIII is one of the least characterized adenoviral protein, very little is known about the function of pVIII in adenovirus life cycle. Earlier work suggested that HAdV-5 pVIII encoded by L4 region is 227 amino acids long (Chroboczek *et al*., 1992) and is present in precursor form in immature virus particles and in cleaved form in mature virions (Chelius *et al*., 2002,Liu *et al*., 2010,Takahashi *et al*., 2006). Crystallographic studies could identify amino acids 31-90 fragment 1 (aa 1-111) and amino acids 163-215 of fragment 2 (aa 158-227) of HAdV-5 pVIII in mature virions (Reddy & Nemerow., 2014).

Recently, we have reported that BAdV-3 pVIII, a protein of 216 amino acids encoded by L6 region (Reddy *et al*., 1998) is localized to the nucleus of the infected cells by binding of pVIII (amino acid 52-72) with importin α-3 (Ayalew *et al*., 2014). The pVIII is expressed as 24 kDa (precursor form) and 8 kDa (cleaved form) proteins in BAdV-3 infected cells. Moreover, while the precursor form (24 kDa) could be detected in empty capsids, the cleaved form (8 kDa) could be detected in mature capsids (Ayalew *et al*., 2014) suggesting that BAdV-3 pVIII appears to be cleaved at potential cleavage site ^{143}LGGG↓S^{147} (Ayalew *et al*., 2014).

Although proteolytic maturation involving cleavage of some structural and non-structural precursor protein is an important aspect of adenovirus life cycle (Mangel & San Martín., 2014,Moyer *et al*., 2015), it is not known what role each individual cleavage event plays in determining adenovirus particle stability and infectivity (Mangel & San Martín., 2014,Moyer *et al*., 2015). The present study was initiated to determine the role of proteolytic cleavage of pVIII in virus assembly, stability and infection. Here, we report that BAdV-3 pVIII is cleaved by adenovirus protease at both potential consensus protease cleavage sites. The cleavage at one consensus protease cleavage sites of pVIII appears essential for the production of

infectious progeny virus. Moreover, cleavage of pVIII at both potential protease cleavage sites is a major contributing factor in determining the stability of adenovirus particles.

5.2 Materials and methods

5.2.1 Cell line and Viruses

Madin Darby Bovine Kidney (MDBK) cells (Kulshreshtha *et al*., 2004), cotton rat lung (CRL) fibroblast cells (Papp *et al*., 1997) and VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) were grown in minimum essential medium (MEM; Sigma Aldrich) supplemented with 10% fetal bovine serum. Human embryo kidney (HEK) 293T cells (ATCC® CRL-3216™) were propagated in Dulbecco's Modified Eagle medium (DMEM) supplemented with 10% FBS. BAV304a (contains BAdV-3 genome from which E3 region is deleted and is replaced by EYFP under CMV promoter) (Du & Tikoo., 2010) and mutant BAdV-3s were propagated in MDBK cells in MEM supplemented with 2% fetal bovine serum.

5.2.2 Antibodies

Polyclonal anti-pVIII serum recognizes a protein of 24 kDa and 8 kDa in BAdV-3 infected cells (Ayalew *et al*., 2014). Production and characterization of sera recognizing DNA binding protein (DBP) (Zhou *et al*., 2001b), pVII (Anand *et al*., 2014), protein V (Kulshreshtha & Tikoo., 2008), hexon (Patel & Tikoo., 2006) and 100K (Makadiya *et al*., 2015) of BAdV-3 have been described. Anti-GFP antibody (Cell Signalling), alexa fluor 680 conjugated goat anti-rabbit antibody (Invitrogen), anti-β-actin monoclonal antibody (Sigma-Aldrich), IRDye800 conjugated goat anti-mouse antibody (Rockland) and alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch) were purchased.

5.2.3 Plasmid construction

Construction of plasmids pDR.bProt, pDR.hProt and pDR.pProt has been described earlier (Makadiya *et al*., 2015). Following plasmids were constructed using standard DNA manipulation techniques.

i) **pEY.pVIII.HA.** PCR was performed using primers p8-c1-fwd + p8-c1-Rev (Table 5.2.1), and plasmid pUC304a+ (Du & Tikoo., 2010) DNA as template to amplify a 704bp DNA fragment. The restriction enzymes *Eco*RI and *Xho*I were used to digest the PCR product. The *XhoI-EcoRI* digested PCR product was ligated to *Xho*I-*EcoR*I digested plasmid pEYFP-C1 (clontech) creating plasmid pEY.pVIII.HA.

ii) **pEY.pVIII.HA143A.** First round of PCR was performed using primer pairs (p8-c1-fwd + p8-143A-Rev) and (p8-143A-fwd + p8-c1-Rev) (Table 5.2.1) and plasmid pEY-pVIII-HA DNA as template to generate 467bp and 281bp products, respectively. The PCR products of first round were mixed and used as templates with primers p8-c1-fwd + p8-c1-Rev to amplify 704bp product by PCR. The restriction enzymes *Eco*RI and *Xho*I were used to digest the PCR product. The *Xho*I-*EcoR*I digested final PCR product was ligated to *Xho*I-*EcoR*I digested plasmid pEYFP-C1creating plasmid pEY.pVIII.HA143A.

iii) **pEY.pVIII.HA108A**. First round of PCR was performed using primer pairs (p8-c1-fwd + p8-DM-Rev) and (p8-DM-fwd + p8-c1-Rev) (Table 5.2.1) and plasmid pEY.pVIII.HA DNA as template to generate 373bp and 386 bp products, respectively. The PCR products of first round were mixed and used as templates with primers p8-c1-fwd + p8-c1-Rev to amplify 704bp product by PCR. The restriction enzymes *Eco*RI and *Xho*I were used to digest the PCR product. The *Xho*I-*EcoR*I digested final PCR product was ligated to *Xho*I-*EcoR*I digested plasmid pEYFP-C1creating plasmid pEY.pVIII.HA108A.

iv) **pEY.pVIII.HA.DM**. First round of PCR was performed using primer pairs (p8-c1-fwd + p8-DM-Rev) and (p8-DM-fwd + p8-c1-Rev) (Table 5.2.1) and plasmid pEY.pVIII.HA143A DNA as a template to generate 373bp and 386bp products, respectively. The PCR products of first round were mixed and used as templates with primers p8-c1-fwd + p8-c1-Rev to amplify 704bp product by PCR. The restriction enzymes *Eco*RI and *Xho*I were used to digest the PCR product. The *Xho*I-*EcoR*I digested final PCR product was ligated to *Xho*I-*EcoR*I digested plasmid pEYFP-C1creating plasmid pEY.pVIII.HA.DM.

v) **pMCS-108A.** First round of PCR was performed using primer pairs (FWD –Reco-p8-del) + Rev –p8-DM-Rev) and (p8-DM-fwd + Rev –Reco-p8-del) and plasmid pMCS + pVIII DNA as template to generate 784bp and 991bp products, respectively. The PCR products of first round were mixed and used as templates with primers FWD –Reco-p8-del + Rev –Reco-p8-del to amplify 1720 bp product by PCR. The restriction enzymes *AscI* and *Afe*I were used to digest the final PCR product. The *AscI* and *Afe*I digested PCR product was ligated to *Asc*I and *Afe*I digested plasmid pMCS+pVIII creating plasmid pMCS-108A.

vi) **pMCS-143A.** First round of PCR was performed using primer pairs (FWD –Reco-p8-del +Rev–p8-143A-Rev) and (p8-143A-fwd + Rev –Reco-p8-del) and plasmid pMCS+pVIII DNA as template to generate 878bp and 886 bp products, respectively. PCR products of first round were mixed and used as templates with primers FWD –Reco-p8-del + Rev –Reco-p8-del to amplify 1720bp product by PCR. The restriction enzymes *AscI* and *Afe*I were used to digest the final PCR product. The *AscI* and *Afe*I digested PCR product was ligated to *Asc*I and *Afe*I digested plasmid pMCS+pVIII creating plasmid pMCS-143A.

vii) **pMCS-DM.** First round of PCR was performed using primer pairs (FWD –Reco-p8-del + Rev –p8-DM-Rev) and (p8-DM-fwd + Rev –Reco-p8-del) and plasmid pMCS-143A DNA as template to generate 784bp and 991 bp products, respectively. The PCR products of first round were mixed and used as templates with primers FWD –Reco-p8-del + Rev –Reco-p8-del to amplify 1720 bp product by PCR. The restriction enzymes *AscI* and *Afe*I were used to digest the final PCR product. The *AscI* and *Afe*I digested PCR product was ligated to *Asc*I and *Afe*I digested plasmid pMCS+pVIII creating plasmid pMCS-DM.

viii) **pUC304a-pVIII-108A.** Restriction enzyme *Xcm*I was used to isolate a 5446 bp DNA fragment from plasmid pMCS-108A. The isolated DNA fragment was used with *Sbf*I digested plasmid pUC304a.dVIII DNA for homologous recombination in E. *coli* BJ5183 to generate plasmid pUC304a-pVIII-108A.

ix) **pUC304a-pVIII-143A.** Restriction enzyme *Xcm*I was used to isolate a 5446 bp DNA fragment from plasmid pMCS-143A. The isolated DNA fragment was used with *Sbf*I digested plasmid pUC304a.dVIII DNA for homologous recombination in E. *coli* BJ5183 to generate plasmid pUC304a-pVIII-143A.

x) **pUC304a-pVIII-DM.** Restriction enzyme *Xcm*I was used to isolate a 5446 bp DNA fragment from plasmid pMCS-DM. The isolated DNA fragment was used with *Sbf*I digested plasmid pUC304a.dVIII DNA for homologous recombination in E. *coli* BJ5183 to generate plasmid pUC304a-pVIII-DM.

5.2.4 Western blotting

Proteins from purified virus, virus infected cell lysates or plasmid DNA transfected cell lysates were run on SDS-PAGE, transferred to PVDF membrane (Bio-Rad) and probed by Western blot using protein specific anti-serum and Alexa Fluor 680, IRDye800 conjugated

antibodies or alkaline phosphatase (AP)-conjugated goat anti-rabbit IgG (Sigma).The membranes probed with fluorophore-conjugated secondary antibody were scanned and analyzed by Odyssey® CLx Imaging System (LI-COR).

Table 5.2.1 List of primers

Primer Name	**Primer sequence**
p8-c1-fwd	TCAGATCTCGAGACATGAGCAAAGAAATTCCCAC
p8-c1-Rev	ACTGCAGAATTCTCAAGCGTAATCTGGAACATCGTATGGGTAGCTATAACCGCTCACAGAG
p8-143A-fwd	CAGATGGAGTCTTTCAACTAGCGGCCGCCTCGCGTTCATCTTTC
p8-143A-Rev	GAAAGATGAACGCGAGGCGGCCGCTAGTTGAAAGACTCCATCTG
p8-DM-fwd	CCTCACATGGCGCTCAAATCGCAGCTGCAGCCGCTGCGGGCGATTACTTTAAAAG
p8-DM-Rev	CTTTTAAAGTAATCGCCCGCAGCGGCTGCAGCTGCGATTTGAGCGCCATGTGAGG

5.2.5 Isolation of mutant BAdV-3

Monolayers of VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) in six- well plates were transfected with 4-6µg of individual plasmid DNA using Lipofectamine 2000 reagent (Invitrogen). At 4hrs post-transfection, the medium was replaced with MEM containing 2% FBS. The cells were observed daily for appearance of any cytopathic effect (CPE). On appearance of CPE, the cells were harvested and freeze thawed three times before using for reinfection of fresh MDBK cells.

5.2.6 CsCl gradient centrifugation

The CsCl gradient centrifugation was carried out as described earlier (Tollefson *et al*., 2007). Briefly, confluent monolayers of MDBK cells were infected with wild-type or

recombinant virus. The cells showing more than 80% cells CPE were collected, pelleted by centrifugation and re-suspended in 5ml medium. The cell lysate was freeze thawed 5 times and was subjected to CsCl density gradient centrifugation at 35000 rpm for 1hr at 4°C. The band containing virus was collected and used for a second round of CsCl density gradient centrifugation at 35000 rpm for 16 hrs at 4°C. The virus was collected, dialyzed against three changes of dialysis buffer to remove traces of cesium chloride and stored in small aliquots at -80°C.

5.2.7 Virus growth kinetics

MDBK cells in 24-well plates were infected with either BAdV304a or BAdV-108A or BAdV-143A at MOI of 1. Infected cells were then collected at different time points (0, 6, 12, 24, 36, and 48 hrs) post-infection, freeze-thawed 3 times and virus in cell lysate was titrated by TCID50 in MDBK cells (Kulshrestha *et al*., 2004).

5.2.8 Virus thermostability assay

The virus thermostability assay was performed as described earlier (Zhao., 2016). Briefly, about 10^5 purified virus particles were incubated at different temperatures (-80°C, -20°C, 4°C, 25°C and 37°C) for 3 days in PBS containing 10% glycerol. Finally, the infectivity of viable virus was determined by $TCID_{50}$. To further assess the thermostability, 10^5 purified virus particles were incubated at different temperatures (-80°C, 4°C, 37°C) for 0, 1, 3 and 7 days in PBS containing 10% glycerol and $TCID_{50}$ assay was performed to determine the remaining infectivity.

5.2.9 Transmission electron microscopy

To analyze CsCl gradient purified BAdV-3 virions by TEM, three hundred mesh grids (Ted Pella, cat# G300) that were previously covered with 0.25% formvar followed by carbon coating (Denton Vacuum Inc.) were suspended for two minutes on to a twenty microliter droplet containing CsCl gradient purified virus particles and fixed with 5% electron microscopy grade glutaraldehyde (catalogue number 16316-10; Electron Microscopy Sciences). After three washes with water, the grids were suspended on a droplet of 0.5% Phosphotungstic Acid (J.B. Em Services Inc. Dorval QC) for one minute. The excess solution was removed and the grids are allowed to dry. Finally, the grids with negatively stained virus particles were examined on an HT7700 transmission electron microscope (Hitachi Inc.) at 80 kv.

To analyse BAdV-3 infected cells by TEM, the monolayers of MDBK cells were infected with either BAdV-304a or BAdV-3-d147-174. At 48 hrs post-infection, the cells were harvested and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at 4°C. Following fixation cells were pelleted in 1% Low melting point agarose, post fixed at room temperature in 1% OsO_4 in 0.1M sodium cacodylate buffer and en bloc stained with saturated Uranyl Acetate in 70% Ethanol. Dehydration was carried out with a graded ethanol series and finalized in propylene oxide (Electron Microscopy Sciences cat#20401). Dehydrated cells were infiltrated with Epon812/Araldite embedding medium by gradually increasing concentration in propylene oxide, and then polymerized in molds with pure Epon/araldite at 60°C for 24 hrs. Finally, the pellet was sectioned with a Reichert ultracut ultramicrotome, each section stained with Reynolds lead citrate and 2% Uranyl acetate and viewed on a HT7700 transmission electron microscope (Hitachi Inc.) at 80 kv.

5.2.10 Fluorescent focus assay

The monolayers of MDBK cells grown in 12 well plates were infected with individual viruses at a MOI of 2. After 18 hrs, GFP positive cells\foci were counted for each well using fluorescent microscope. The average number of GFP positive cells was quantified as fluorescent focus unit (FFU) per field.

5.2.11 TEM analysis of subcellular distribution of purified viruses

The monolayers of MDBK cells in one well of 6 well plate were incubated with individual viruses at a MOI of 10 at 4°C. After 1 hr of incubation, the cells were washed before incubating again at 37°C. After incubating for 30 mins, the cells were then processed for TEM analysis as described earlier (Kulshreshtha *et al.,* 2004).

5.2.12 Viral genome replication

Viral genome replication was measured as described (Wu *et al*., 2012) with minor modifications. Briefly, MDBK cells were infected with BAdV304a, BAdV-108A or BAdV-143A and harvested at indicated times post infection. The infected cells were pelleted, washed one time with phosphate-buffered saline (PBS) and then re-suspended in isotonic buffer. After incubating the cells on ice for 10 min, the nuclei were then pelleted by centrifugation at 2000 × g at 4°C for 5 min. The pelleted nuclei were suspended in 200 µl PBS and used for DNA extraction using a DNeasy blood and tissue kit (Qiagen) following manufacturer's instructions. Finally, the DNA was re-suspended in double distilled water and subjected to quantitative PCR using Bio-Rad real time PCR system. The two sets of primers used for quantitative PCR were primer pair Q adeno Fwd and Q adeno Rev and primer pair Actin Fwd and Actin Rev (Table 3.2.1). Genome replication was calculated as the value of viral genome copy number divided by

actin copy number. For comparison, the value of each virus was normalized to that of BAV304a at 6hpi and shown as relative viral genome copy number.

5.2.13 Statistical analysis

All data were analyzed using GraphPad Prism version 6 (GraphPad Software, Inc., La Jolla, CA, USA). Statistical differences among the groups were calculated using unpaired t-test. Differences were considered significant at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

5.3 Results

5.3.1 Analysis of the pVIII protein sequences for potential protease cleavage sites

The adenovirus protease recognizes and cleaves at two consensus cleavage motifs (M/I/L)XGX-G and(M/I/L)XGG-X (where X is any amino acid) (Webster *et al*., 1989). Earlier, analysis of amino acid sequence of BAdV-3 pVIII (Ayalew *et al*., 2014) revealed two potential adenoviral protease cleavage sites (Fig.5.3.1A), which are conserved with potential protease cleavage sites in pVIII encoded by human adenovirus 5 (HAdV-5) or porcine adenovirus 3 (PAdV-3) (Fig.5.3.1B). In contrast, analysis of pVIII encoded by HAdV-5 and PAdV-3 revealed the presence of three potential protease cleavage sites (Fig.5.3.1B).

5.3.2 Analysis of cleavage of BAdV-3 pVIII at potential protease cleavage site(s)

To determine whether pVIII is cleaved at one or both potential protease cleavage sites, we constructed plasmid pEY.pVIII.HA (expressing EYFP and HA tagged pVIII) (Fig.5.3.2A). The 293T cells were co-transfected with plasmids pEY.pVIII.HA + plasmid pDsRed-C1 DNAs or plasmid pEY.pVIII.HA + pDR.bProt (expressing DsRed tagged BAdV-3 protease) DNAs. After 48 hrs post transfection, the cells were collected lysed and the lysates were analysed by Western blotting using anti-GFP serum. As seen in Fig.5.3.2 anti-GFP serum detected a protein of 52 kDa

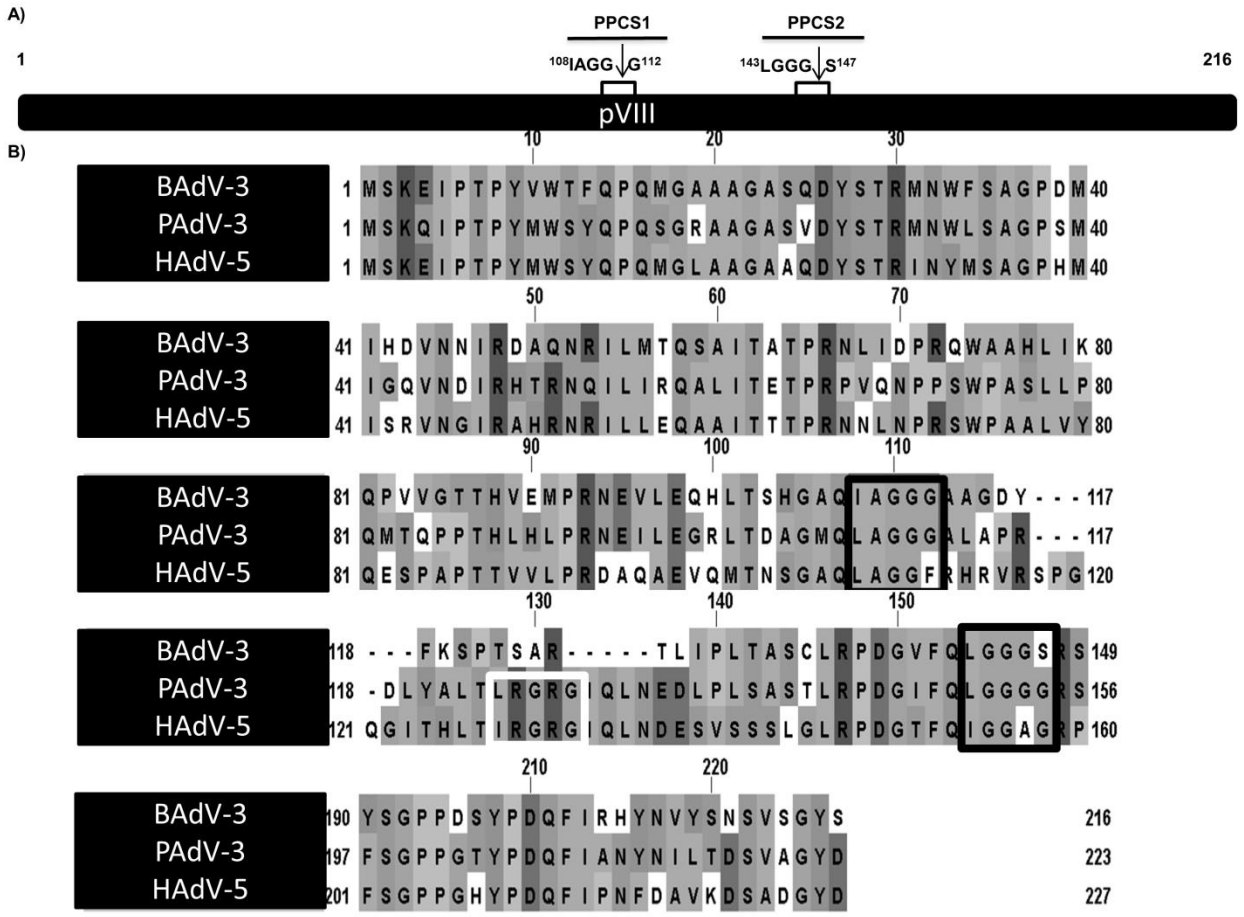


Figure 5.3.1 Consensus Protease cleavage sites of pVIII. (A). Schematic diagram of BAdV-3 pVIII showing the two potential protease cleavage sites (PPCS). PPCS1 (amino acid 108-112); PPCS2 (amino acids 143-147). Arrows depict the site of cleavage. **(B). Conservation of PPCS in pVIII protein.** Black frames indicate the adenovirus protease consensus cleavage site present in pVIII protein of BAdV-3, PAdV-3 and HAdV-5 where as white frame indicate the AVP consensus cleavage site present in pVIII protein of PAdV-3 and HAdV-5 only. Sequences were downloaded from Uniprot (BAdV-3 Uniprot Accession # O92788; PADV-3# Q83453; HAdV-5 #P24936) and aligned with TCofee (Notredame *et al*., 2000). The figure was created using JalView (Waterhouse *et al*., 2009).

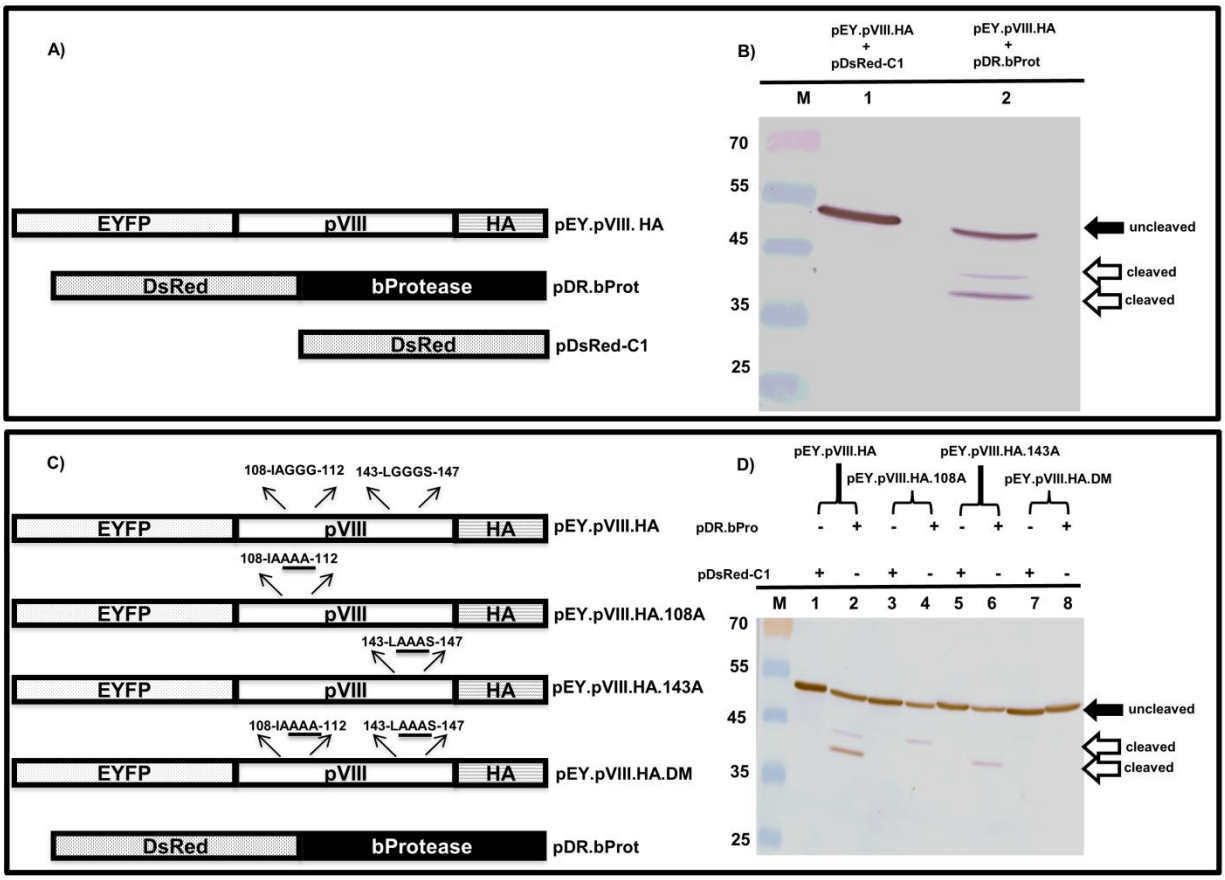


Figure 5.3.2 Analysis of cleavage of BAdV-3 pVIII protein in transfected 293T cells. **(A). Schematic diagram of plasmid DNAs.** The name of the plasmids is indicated on the right of diagrams. The EYFP (enhanced yellow fluorescent protein), pVIII (BAdV-3), protease (BAdV-3) and DsRed DNA are indicated. **(B). Western blot.** Cell lysates were prepared from 293T cells co-transfected with the indicated plasmid DNA. The proteins from the prepared lysates were run on 12 % SDS-PAGE, transferred to PVDF membrane and analysed by Western blotting using anti-GFP antibody. Solid arrows indicate uncleaved EY.pVIII.HA fusion protein and open arrows indicate the cleaved product. The molecular mass markers (M) in kDa are shown on the left. **(C). Schematic diagram of plasmid DNAs showing potential protease cleavage sites**. The origin of DNA is depicted. The amino acid sequence of potential protease cleavage sites is

depicted on the top of plasmid pEY.pVIII.HA. The glycine to alanine substitution in predicted protease cleavage sites are underlined and depicted on top of plasmids pEY.pVIII.HA108A, pEY.pVIIII.HA143A and pEY.pVIII.HA.DM. The name of the plasmid is on right. **(D)**. **Cleavage of BAdV-3 pVIII mutant proteins in transfected cells.** Cell lysates were prepared from 293T cells co-transfected with the indicated plasmid DNA's. The proteins from the prepared lysates were run on 12 % SDS-PAGE, transferred to PVDF membrane and analysed by Western blotting using anti-GFP antibody. Solid arrows indicate uncleaved fusion protein and open arrows indicate the cleaved product. The molecular mass markers (M) in kDa are indicated on the left.

(corresponding to EYFP-pVIII-HA fusion protein) in the cells co-transfected with plasmid pEY.pVIII.HA + pDsRed-C1 DNAs. In contrast, anti-GFP serum detected proteins of 52 kDa, 40 kDa (corresponding to cleavage product EYFP-pVIII amino acid 1 to 111 fusion protein) and 43 kDa (corresponding to cleavage product EYFP-pVIII amino acid. 1 to 146 fusion protein) in the cells co-transfected with plasmid pEY.pVIII.HA + pDR.bProt DNAs, suggesting that pVIII is cleaved at both potential cleavage sites and that both sites are active.

To further characterize the cleavage of pVIII by adenoviral protease, we constructed three additional plasmids (pEY.pVIII.HA.108A, pEY.pVIII.HA.143A and pEY.pVIII.HA.DM) (Fig.5.3.2C). Plasmid pEY.pVIII.HA.108A contain substitution of glycines at amino acid position 110, 111 and 112 of BAdV-3 pVIII to alanine; plasmid EY.pVIII.HA143A contain substitution of glycines at position 144, 145 and 146 of BAdV-3 pVIII to alanine and plasmid pEY.pVIII.HA.DM contain substitution of glycines at position 110, 111, 112, 145 and 146 of BAdV-3 pVIII to alanine. Identity of these plasmid DNAs was confirmed by DNA sequencing. The 293T cells were co-transfected with each of these plasmids DNA along with either plasmid pDsRed-C1 DNA or plasmid pDR.bProt DNA. After 48 hrs post transfection, the cells were collected, lysed, and the lysates were analysed by Western blotting using anti-GFP serum. As seen in Fig.5.3.2D anti-GFP serum detected a protein of 52 kDa in the lysates of the cells co-transfected with plasmid pEY.pVIII.HA + pDsRed-C1 (lane 1), pEY.pVIII.HA.108A + pDsRed-C1b(lane 3), pEY.pVIII.HA143A + pDsRed-C1 (lane 5), pEY.pVIII.HA.DM + pDsRed-C1 and pEY. pVIII.HA.DM + pDR.bProt (lane 7) DNAs suggesting that pVIII is not cleaved in absence of protease. As seen earlier, anti-GFP serum detected proteins of 52 kDa, 43 kDa and 40 kDa in the lysates of cells co-transfected with plasmid pEY.pVIII.HA+ pDR.bProt DNAs (lane 2). However, anti-GFP serum detected proteins of 52 kDa and 43 kDa in the lysates of the cells co-

transfected with plasmid pEY.pVIII.HA.108A + pDR.bProt DNAs DNA (lane 4). Similarly, anti-GFP serum detected two proteins of 52 kDa and 40 kDa in the lysates of the cells co-transfected with plasmid pVIII.HA.143A + pDR.bProt DNAs (lane 6). In contrast, anti-GFP serum detected only a protein of 52 kDa in the lysates of the cells co-transfected with plasmid pEY.pVIII.HA.DM + pDR.bProt DNAs (lane 8).

5.3.3 Cleavage of BAdV-3 pVIII by protease encoded by other *Mastadenoviruses*

To determine if BAdV-3 pVIII protein can be cleaved by proteases encoded by other *Mastadenoviruses*, the 293T cells were co-transfected with plasmids pEY.pVIII.HA and either plasmid pDsRed-C1 or plasmid pDR.bProt (expressing BAdV-3 protease) or plasmid pDR.hProt (expressing human adenovirus 5 [HAdV-5] protease) or plasmid pDR.pProt (expressing porcine adenovirus 3 [PAdV-3] protease) DNAs. After 48 hrs post transfection, the cells were collected, lysed and the lysates were analysed by Western blotting using anti-GFP serum. As seen in Fig.5.3.3A, anti-GFP serum detected a protein of 52 kDa (uncleaved EYFP-pVIII-HA fusion protein) in the cells co-transfected with plasmid pEY.pVIII.HA and pDsRed-C1 DNAs (lane 1). In contrast, anti-GFP serum detected proteins of 52kDa (uncleaved EYFP-pVIII-HA fusion protein), 43kDa (cleavage product EYFP-pVIII amino acid 1 to 146 fusion protein) and 40kDa (cleavage product EYFP-pVIII amino acid 1 to 111 fusion protein) in the cells co-transfected with plasmid pEY.pVIII.HA and either pDR.bProt (lane 2) pDR.hProt (lane 3) or pDR.pProt (lane 4) DNAs, indicating that BAdV-3 pVIII can be cleaved at both potential cleavage sites by protease encoded by HAdV-5 and PAdV-3.

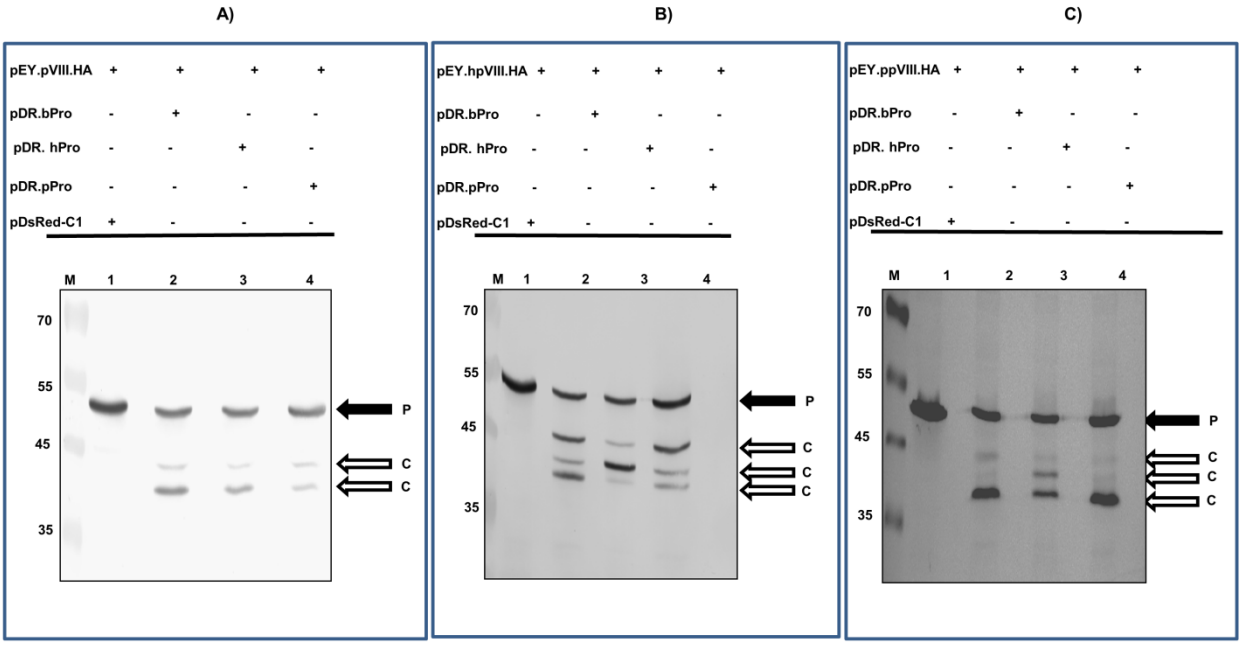


Figure 5.3.3 Cleavage of pVIII by different adenoviral proteases in transfected cells. **(A-C).** Cell lysates were prepared from 293T cells co-transfected with the indicated plasmid DNA's. The proteins from the prepared lysates were run on 12 % SDS-PAGE, transferred to PVDF membrane and analysed by Western blotting using anti-GFP antibody. The cleavage of pVIII of **(A)** BAdV-3, **(B)** HAdV-5 and **(C)** PAdV-3 by different adenoviral proteases was analysed. Solid arrows indicate uncleaved fusion protein and open arrows indicate the cleaved product. The molecular mass markers (kDa) are indicated on the left of each panel.

5.3.4 Cleavage of pVIII protein encoded by other *Mastadenoviruses*

Analysis of amino acid sequence of pVIII of HAdV-5 and PAdV-3 revealed three potential protease cleavage sites. To analyze the cleavage of pVIII protein of HAdV-5 and PAdV-3, we constructed plasmids expressing pVIII protein of HAdV-5 (pEY.hpVIII.HA) or PAdV-3 (pEY.ppVIII.HA) as EYFP fusion protein. The 293T cells were co-transfected with DNA's from plasmid pEY.hpVIII.HA and plasmid pDsRed-C1 (control), pDR.bProt (expressing BAdV-3 protease), pDR.hProt (expressing HAdV-5 protease) or pDR.pProt (expressing PAdV-3 protease). After 48 hrs post transfection, the cells were collected, lysed and the cell lysate were analysed by Western blotting using anti-GFP serum. As seen in Fig.5.3.3B anti-GFP serum detected a protein of 53 kDa (uncleaved EYFP-hpVIII-HA fusion protein) in the cells co-transfected with plasmid pEY.hpVIII.HA and pDsRed-C1 DNAs (lane 1). In contrast, proteins of 53 kDa, 40 kDa (cleavage product EYFP-hpVIII amino acids 1 to 111 fusion protein), 42 kDa (cleavage product EYFP-hpVIII amino acids 1 to 131 fusion protein) and 45 kDa (cleavage product EYFP-hpVIII amino acids 1 to 157 fusion protein) were detected in the lysates of the cells co-transfected with plasmid pEY.hpVIII.HA and either pDR.bProt (lane 2)or pDR.hProt (lane 3) or pDR.pProt (lane 4) DNAs, indicating that HAdV-5 pVIII can be cleaved at all three potential cleavage sites by protease encoded by HAdV-5 , BAdV-3 or PAdV-3. Interestingly, the 45kDa band and 40 kDa bands were thicker than 42 kDa band in pEY.hpVIII.HA and pDR.bProt co-transfected cells (Fig.5.3.3B, lane2). Similarly, the 45kDa band was thicker than the 42kDa and 40kDa band in pEY.hpVIII.HA and pDR.pProt co-transfected cells (Fig.5.3.3B, lane 4). However, the 42kDa band was thicker than both 45kDa and 40kDa bands in pEY.hpVIII.HA and pDR.hProt co-transfected cells (Fig.5.3.3B, lane 3).

Similarly, anti-GFP serum detected a protein of 53 kDa (uncleaved EYFP-ppVIII-HA fusion protein) in the cells co-transfected with plasmid pEY.ppVIII.HA and pDsRed-C1 DNAs (lane 1). In contrast, proteins of 53 kDa, 40 kDa (cleavage product EYFP-ppVIII amino acids1 to 111 fusion protein), 42 kDa (cleavage product EYFP-ppVIII amino acids 1 to 131 fusion protein) and 45 kDa (cleavage product EYFP-ppVIII amino acids 1 to 157 fusion protein) were detected in lysate of the cells co-transfected with plasmid pEY.ppVIII.HA and either plasmid pDR.bProt (lane 2) or plasmid pDR.hProt (lane 3) or plasmid pDR.pProt (lane 4) DNAs, indicating that PAdV-3 pVIII can also be cleaved at all three potential cleavage sites by protease encoded by HAdV-5 , BAdV-3 or PAdV-3 (Fig.5.3.3C).

5.3.5 Construction of BAdV-3 expressing pVIII protein with altered protease cleavage site(s)

To determine the role of cleavage of pVIII protein in BAdV-3 replication, we decided to generate recombinant BAdV-3 expressing protease cleavage site(s) mutant pVIII protein. To achieve this, we constructed three different plasmids containing mutant full length genomic clone of BAdV-3 (Fig.5.3.4A). The plasmid pUC304a-pVIII-108A contained a BAdV-3 pVIII gene with substitution of amino acid 110, 111 and 112 from glycine to alanine in potential protease cleavage site I of the protein. The plasmid pUC304a-pVIII-143A contained a BAdV-3 pVIII gene with substitution of amino acid 144, 145 and 146 from glycine to alanine in potential protease cleavage site II of the protein. Similarly, plasmid pUC304a-pVIII-DM, contained BAdV-3 pVIII gene with substitution of amino acid 110, 111, 112, 144, 145 and 146 from glycine to alanine in potential protease cleavage site I and site II of the protein.

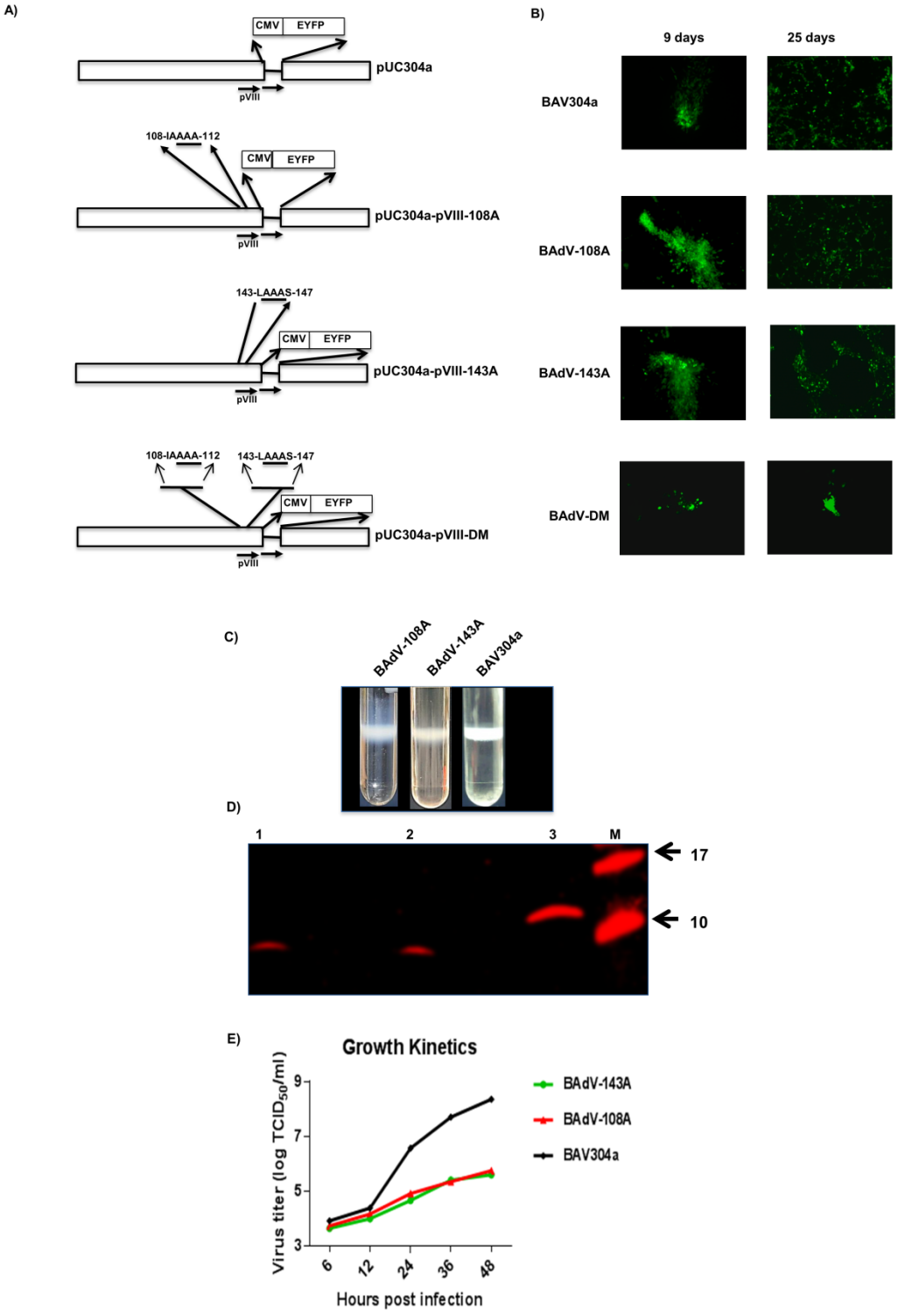


Figure 5.3.4 Isolation and characterization of mutant BAdV-3. (A). Schematic representation of plasmids. The BAdV-3 sequence is represented by hollow box. The thin line

represents deleted E3 region (Zakhartchouk *et al*., 1998b). The alanines substituted for glycines are underlined. The numbers indicate the amino acids of BAdV-3 pVIII, CMV (human cytomegalo virus immediate early promoter); EYFP (enhanced yellow fluorescent protein). Arrows indicate the direction of the transcription. The black box represents the nucleotide sequence of BAdV-3 pVIII. **(B). Direct fluorescence.** Monolayer of VIDO DT1 cells (Du & Tikoo., 2010) were transfected with 4-6 μg of plasmid DNA; pUC304a (BAV304a), pUC304a-pVIII-108A (BAdV-108A), pUC304a-pVIII-143A (BAdV-143A, or pUC304a-pVIII-DM (BAdV-DM) and visualized at indicated times post transfection for the expression of EYFP and development of cytopathic effects using fluorescent microscope TCS SP5 (Leica). Increase in green fluorescence indicating virus production could be seen after 9th day post transfection. **(C). CsCl purification.** Virus was purified from infected cell lysates by $CsCl_2$ density gradient purification. Mature virion bands after double CsCl density gradient centrifugation are shown. **(D). Western blot.** Proteins from purified BAV304a (lane 1), BAdV-108A (lane 2) or BAdV-143A (lane 3) were run on 4-20% gradient SDS-PAGE, transferred to PVDF and probed in Western blot using anti-pVIII serum (Ayalew *et al*., 2014). **(E). Virus growth kinetics.** The infected MDBK cells were collected at indicated times post infection, freeze-thawed and virus was titrated on MDBK cells. BAV304a (♦), BAdV-108A (▲), BAdV-143A (●).

To isolate mutant BAdV-3s, VIDO DT1 cells (cotton rat lung fibroblasts expressing I-SceI protein) (Du & Tikoo., 2010) were transfected with individual plasmid (pUC304a-pVIII-108A, pUC304a-pVIII-143A or pUC304a-pVIII-DM) DNAs using lipofectamine 2000 reagent. The transfected cells were observed daily for development of the cytopathic effects (CPE). The cells transfected with either plasmid pUC304a-pVIII-108A or pUC304a-pVIII-143A DNAs showed increase in green fluorescence 9th day post transfection indicating virus production (Fig.5.3.4B). The cells showing cytopathic effects were harvested 25 day post transfection, freeze thawed three times and used to purify virus using CsCl density gradient (Tollefson *et al*., 2007). The recombinant viruses were named BAdV-108A (pUC304a-pVIII-108A) and BAdV-143A (pUC304a-pVIII-143A).

Although fluorescent focus forming units could be observed after 9 day post transfection in VIDO DT1 cells transfected with plasmid pUC304a-pVIII-DM DNA, however, these fluorescent foci gradually disappeared without showing any observable cytopathic effect (Fig.5.3.4B).

The BAV304a, BAdV-108A and BAdV-143A were propagated in MDBK cells and purified by double CsCl density gradients (Fig.5.3.4C). The number of MDBK cells used to purify BAdV-108A and BAdV-143A was twice the number of MDBK cells used to purify BAV304a. To determine the incorporation of pVIII in purified virions, proteins from purified virions were separated by 4-20% gradient SDS-PAGE, transferred to PVDF and probed in Western blot using anti-VIIIb serum. As expected (Fig.5.3.4D), anti pVIIIb detected a protein of 8 kDa in purified BAV304a (lane 1) and BAdV-108A (lane 2), and a protein of 12 kDa in purified BAdV-143A (lane 3).

5.3.6 Virus growth kinetics

To determine if the mutation of protease cleavage sites of pVIII affects BAdV-3 replication, we compared the growth kinetics of BAdV-304a, BAdV-108A and BAdV-143A. The monolayers of MDBK cells in 24-well plates were infected with either CsCl purified BAV304a, BAdV-108A or BAdV-143A at MOI of 1. At different times post infection (6, 12, 24, 36, 48 hrs), the infected cells were collected, freeze-thawed three times and virus in cell lysate was titrated by $TCID_{50}$ in MDBK cells (Kulshreshtha et al., 2004). As seen in Fig.5.3.4E, BAV304a grew to a titer of $10^{8.4}$ $TCID_{50}$/ml. However, BAdV-108A and BAdV-143A could only grow to a titer of $10^{5.75}$ and $10^{5.6}$ $TCID_{50}$/ ml, respectively.

5.3.7 Virus infectivity and genome replication

To determine the infectivity of mutant viruses, GFP expression was used as an indicator to determine the virus particles infectivity in a single round infection assay. As seen in Fig.5.3.5A, there was no significant difference in the number of GFP expressing cells when MDBK cells infected with equal amount of infectious virus were analysed after 18 hrs post infection.

To determine if the alteration of protease cleavage of pVIII modulate the replication of mutant viruses, we determined the mutant viral DNA replication by quantitative real time PCR as described in materials and methods. As seen in Fig.5.3.5B during the early phase of infection (6hpi and 12hpi) virus genome replication appeared similar in different viruses. However, during late phase of infection (24hpi and 36hpi) BAV-304a and BAdV-108A replicated marginally better than BAV304a or BAdV-143A. Similarly, BAV304 replicated marginally better than BAdV-143A.

To determine if alteration of protease cleavage of pVIII modulate the release of the virus from endosomes, MDBK cells infected with equal amounts of individual infectious viruses were

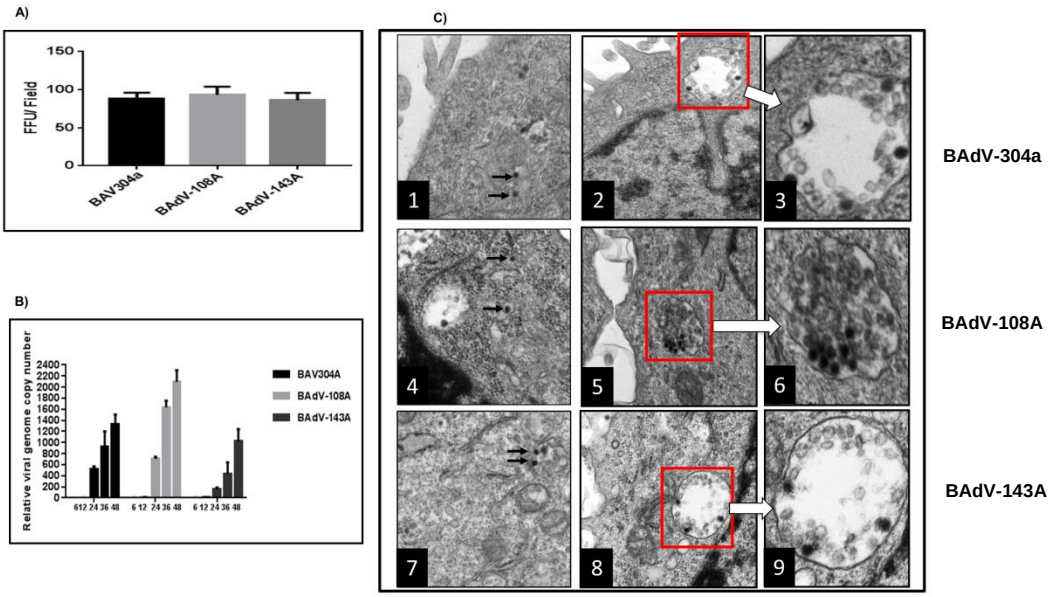


Figure 5.3.5 Analysis of mutant BAdV-3s. (A). Virus infectivity. The MDBK cells were infected with equivalent amounts of infectious particles of indicated virus. The infected cells were visualized for the expression of GFP at 18 hrs post infection by fluorescent microscope TCS SP5 (Leica). **(B). Viral genome replication.** The MDBK cells were infected with equivalent amounts of infectious particles of indicated virus in triplicate. At indicated times post infection, cells were harvested, and genomic DNA was isolated. The viral genome copy number was determined by quantitative PCR and divided by actin copy number for normalization. For comparison the normalized genome copy number values for each virus at each time point were compared to that of BAV304a virus at 6hpi **C). Sub cellular distribution of BAdV-3.** Monolayers of MDBK cells (1×10^6 cells/well) were incubated with 1.4×10^7 purified virions at 4°C. After 1 hr of incubation, the cells were incubated at 37°C for 30 min. Finally, the cells were processed and visualized by transmission electron microscopy. For each virus ten cells were selected randomly and virus particles in endosomes, cytoplasm and at plasma membrane were counted. BAV304a (panel 1-3), BAdV-108A (panel 4-6), BAdV-143A (panel 7-9).

analyzed by TEM after 90 min of infection. As seen in Fig.5.3.5C, no significant difference could be observed in the number of virus particles in the endosome or cytosol between BAV304a, BAdV-108A or BAdV-143A at 90 min pi.

5.3.8 Thermostability of virus

To determine if mutation of protease cleavage site of pVIII protein alters the stability of mutant BAdV-3, we compared the thermostability of BAdV-304a and BAdV-108A and BAdV-143A. About 10^5 purified virus particles were incubated at different temperatures (-80°C, -20°C, 4°C, 25°C and 37°C) for 3 days in PBS containing 10% glycerol. Finally, the infectivity of viable virus was determined by $TCID_{50}$. As seen in Fig.5.3.6A., there was not much decrease in virus titer when viruses were incubated at -80°C, -20°C, 4°C and 25°C for 3 days. However, the virus titers decreased significantly when viruses were incubated at 37°C for 3 days. Compared with BAdV-304a, the drop in the titre was more for BAdV-108A and BAdV-143A (Fig.5.3.6A). To further assess the thermostability, 10^5 purified virus particles were incubated at different temperatures (-80°C, 4°C, 37°C) for 0, 1, 3 and 7 days in PBS containing 10% glycerol measuring the remaining infectivity by $TCID_{50}$. As seen in Fig.5.3.6B, C and D, when incubated at 37°C more rapid loss of infectivity was observed for BAdV-108A and BAdV-143A. While BAdV-108A and BAdV-143A lost the infectivity within 5 days BAV-304a lost the infectivity only after 7days incubation at 37°C.

5.3.9 Analysis of protein expression in infected cells

To analyze if there is any difference in expression of viral protein, MDBK cells were infected with either BAV-304a or BAdV-108A or BAdV-143A. After 24 hrs post infection, the cells were collected, lysed and the lysates were analysed by Western blotting using protein

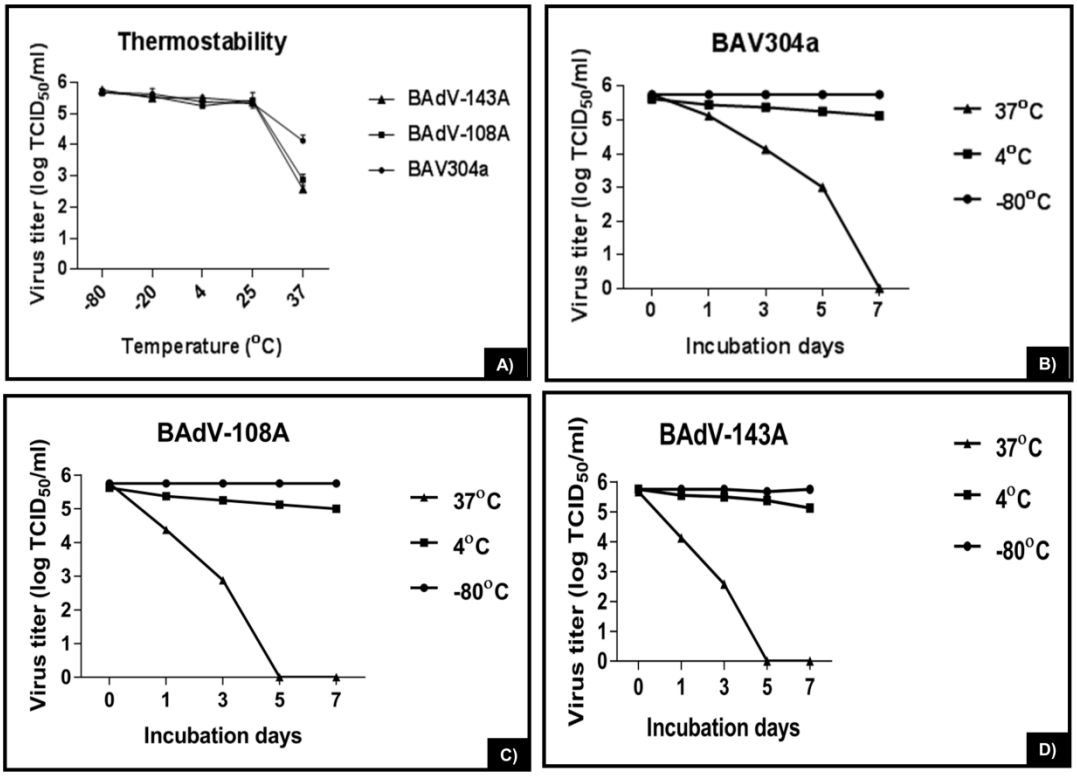


Figure 5.3.6 Thermostability of mutant virions. **(A)**. Purified virions grown in MDBK cells (10^5 infectious virus particles (TCID50) were incubated at various temperatures for 3 days in PBS containing 10% glycerol and the residual viral infectivity was determined by titration on MDBK cells. **(B)** Purified BAV304a (10^5 TCID50) grown in MDBK cells were incubated at different temperatures for indicated periods of time in PBS containing 10% glycerol and the residual viral infectivity was determined by titration on MDBK cells. **(C).** Purified BAdV-108A (10^5 TCID50) grown in MDBK cells were incubated at different temperatures for indicated periods of time in PBS containing 10% glycerol and the residual viral infectivity was determined by titration on MDBK cells. **(D).** Purified BAdV-143A (10^5 TCID50) grown in MDBK cells were incubated at different temperatures for indicated periods of time in PBS containing 10% glycerol and the residual viral infectivity was determined by titration on MDBKcells. The data is representative of two independent experiments.

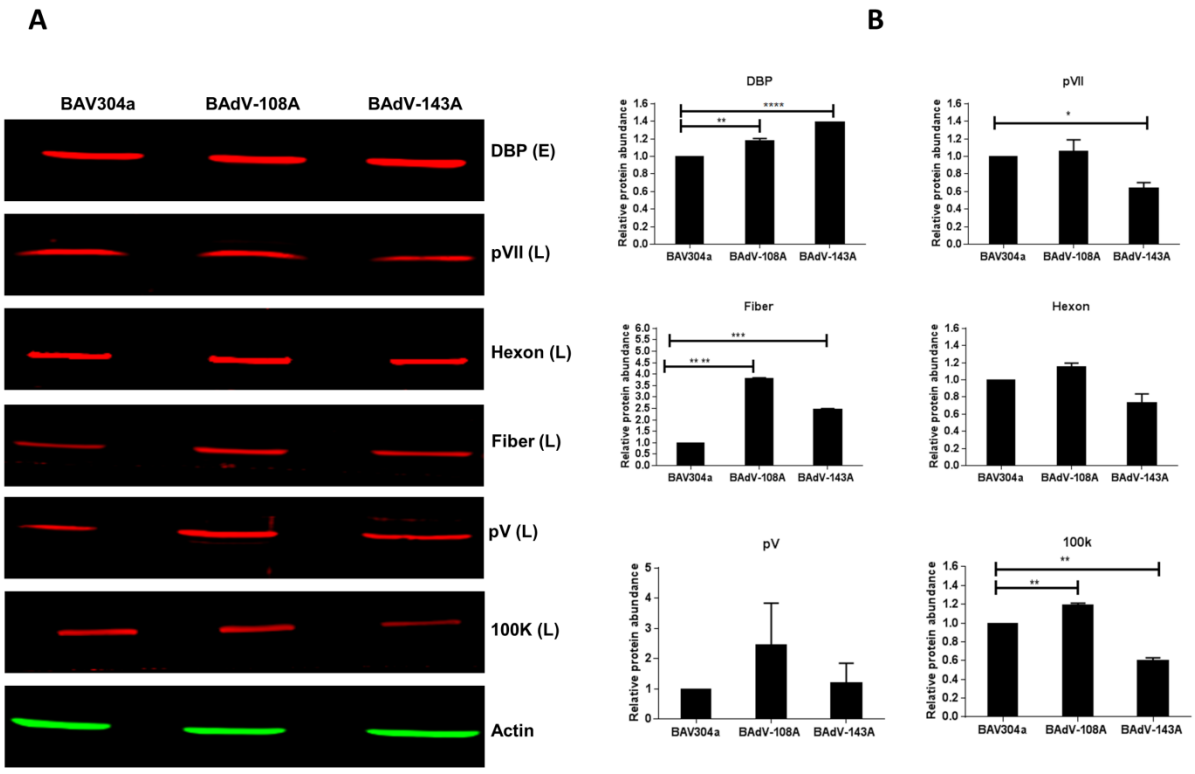


Figure 5.3.7 Analysis of viral protein expression in infected cells. Cell lysates were prepared from infected MDBK cells. Proteins from the prepared cell lysates were run on 4-20% gradient SDS-PAGE, transferred to PVDF membranes and probed by Western blot using anti-DNA binding protein (DBP) (Zhou *et al*., 2001b), anti-pVII (Anand *et al*., 2014), anti-proteinV (Kulshreshtha & Tikoo., 2008), anti- hexon (Patel & Tikoo., 2006) and anti-100K (Makadiya *et al*., 2015) sera followed by Alexa Fluor 680 conjugated goat anti-rabbit antibody (Invitrogen). β-actin (G) was detected by Western blot using mouse anti-β-actin monoclonal antibody (Sigma-Aldrich) followed by IRDye800 conjugated goat anti-mouse antibody (Rockland). The name of the proteins is depicted on the right of the panel. DBP (DNA binding protein). Early (E), Late (L). (b) The results were analyzed by using Odyssey Infrared Imaging System. Values represent averages from two independent repeats and error bars indicate the standard deviations.

specific rabbit antisera. As seen in Fig.5.3.7A, B no appreciable decrease could be detected in the expression of viral proteins in BAdV-108A infected cells compared to BAV-304a infected cells. Interestingly, expression of all tested proteins appeared increased in BAdV-108A infected cells. In contrast compared to BAV304a, though expression of early viral protein (DBP) and late protein fiber appears increased in BAdV-143A infected cells, the expression of some of the viral late proteins (pVII, 100K and hexon) appears reduced in BAdV-143A infected cells.

5.3.10 Analysis of protein incorporation in viral particles

To analyse the incorporation of viral protein in the progeny virions, the proteins from purified virions were run on SDS-PAGE, transferred to PVDF membrane and probed in Western blot using viral protein specific serum. As seen in Fig.5.3.8A, B, compared to BAV304a, no significant decrease could be observed in the incorporation of the viral proteins in purified BAdV-108A or BAdV-143A virions.

5.3.11 Transmission Electron Microscopy

To assess whether mutation of single protease cleavage site of pVIII protein has any effect on virus structure BAV304a , BAdV-108A or BAdV-143A were propagated in MDBK cells and purified by $CsCl_2$ density gradient (Fig.5.3.9A) as described in materials and methods. The virus particles were then observed by negative-staining electron microscopy. As seen in Fig.5.3. 9A the BAV304a particles (panel 1-2) had typical icosahedral structure and most of them were intact. However, unlike the BAV304a particles, BAdV-108A (panel 3 -4) or BAdV-143A (panel 5-6) virus particles appeared more rounded disrupted capsids and lacking capsid fragments.

To further assess if the mutation of single protease cleavage site of pVIII protein affect the formation of BAdV-3 particles, transmission electron microscopy was done on MDBK cells infected with BAdV304a, BAdV-108A or BAdV-143A. The infected cells were harvested

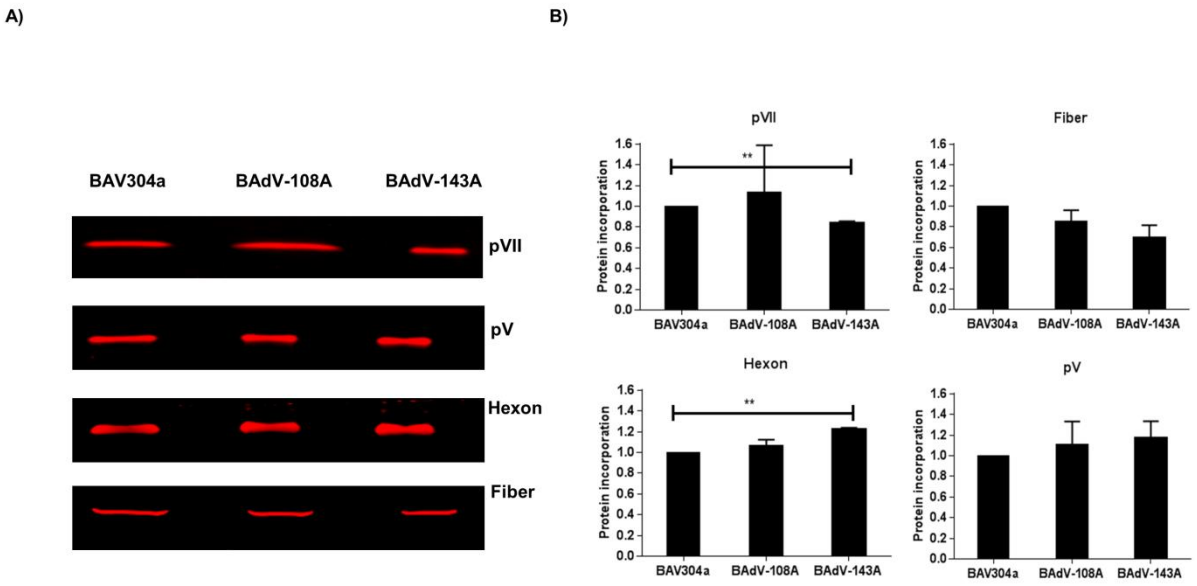
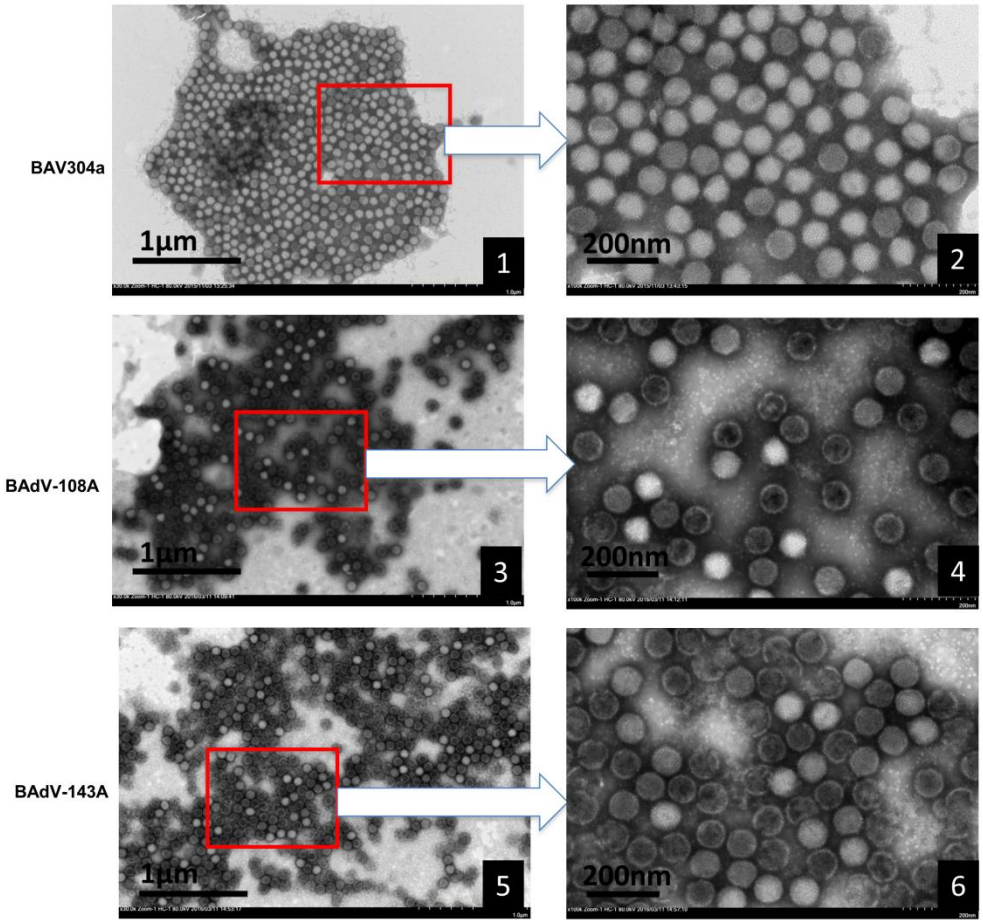


Figure 5.3.8 Analysis of viral protein incorporation in purified virions. Proteins from double $CsCl_2$ purified BAV304a, BAdV-108A or BAdV-143A virions were run on 4-20% gradient SDS-PAGE, transferred to PVDF membrane and probed in Western blot using protein specific antisera. The name of the detected protein is indicated on the right of the panel.

A)


BAV304a
1µm
1
200nm
2
BAdV-108A
1µm
3
200nm
4
BAdV-143A
1µm
5
200nm
6

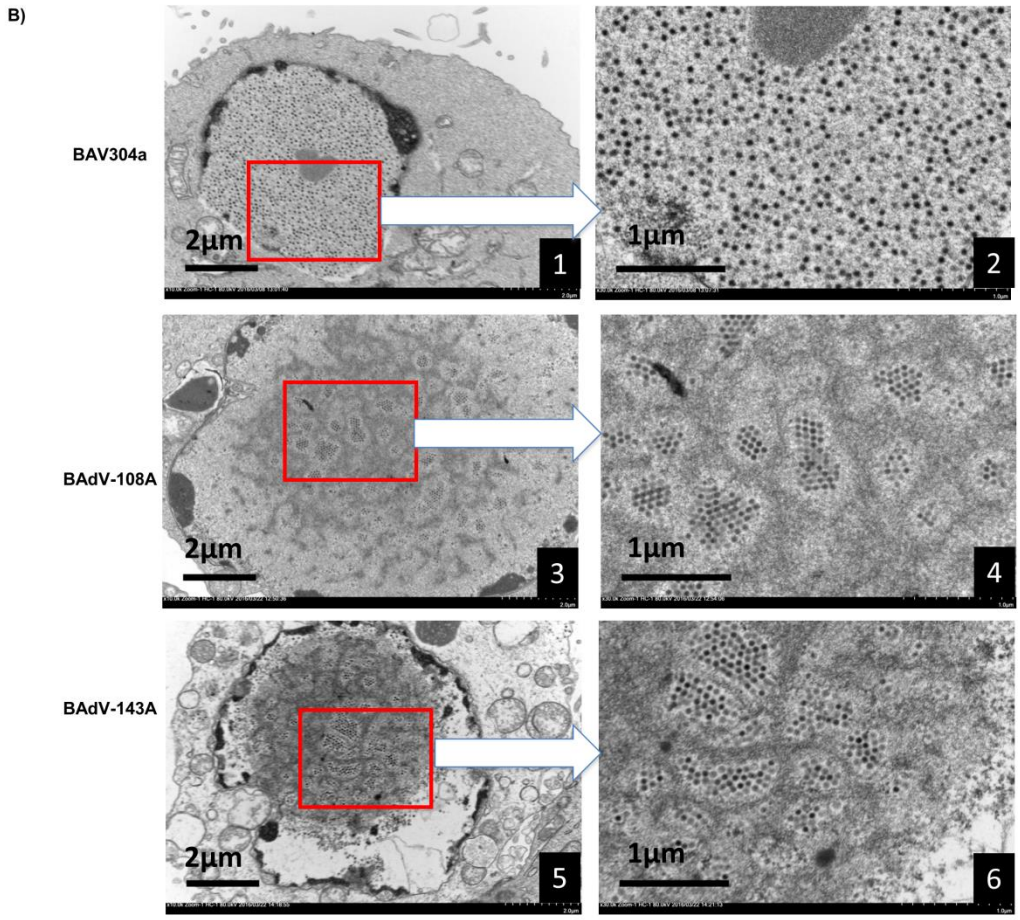


Figure 5.3.9 Electron microscopic analysis. (A). Purified BAV304a (panel 1), BAdV-108A (panel 3) or BAdV-143A (panel 5) (Magnification 30000×). The arrows depict the enlargement of selected boxed region of panel 1 (panel 2), panel 3 (panel 4) and panel 5 (panel 6) (Magnification 1000000×). **(B).** BAV304a infected (panel 1) or BAdV-108A infected (panel 3) and BAdV-143A (panel 5) infected MDBK cells (magnification 10000×). The arrows depict the enlargement of selected boxed region of panel 1 (panel2), panel 3 (panel 4) and panel 5 (panel 6) (Magnification 30000×).

48 hrs post infection and examined by transmission electron microscopy. As seen in Fig.5.3.9B BAV304a particles were uniformly distributed and loosely arranged (panel1-2). However, BAdV-108A (panel 3-4) and BAdV-143A (panel 5-6) particles were clustered together in groups and appeared tightly organized in rows.

5.3.12 BAdV-3 pVIII complements the defect of BAV.pVIIIDM

In order to determine if cleavage of pVIII at both potential protease consensus sites is essential for viral replication, we performed complementation assay. VIDO DT1 cells in 6 well plates were co-transfected with plasmid (pUC304a-pVIII-DM + pC-pVIII; or pUC304a-pVIII-DM + pCDNA3) DNAs (Fig.5.3.10A). The transfected cells were observed under fluorescent microscope daily for the appearance of fluorescent focus forming units. As seen in Fig.5.3.10B Co-transfection of VIDO DT1 cells with plasmid pUC304a-pVIII-DM and pCDNA3 DNAs did not resulted in progeny virus production as indicated by absence of any increase in the number of FFU from day 5 to day 11 post transfection (Fig.5.3.10B). However, co-transfection of VIDO DT1 cells with plasmid pUC304a-pVIII-DM and pC-pVIII DNAs resulted in production of progeny virus as indicated by significant increase in number of FFU from day 5 to day 11 post transfection (Fig.5.3.10B).

5.4 Discussion

The final step in adenovirus morphogenesis involves the maturation of immature virus particle by proteolytic cleavage of three minor capsid proteins, three core proteins and one non-structural protein (Mangel & San Martín., 2014). The major player in this complex process is 23 kDa adenovirus protease, which recognizes consensus motifs (M/I/L)XGX-G and (M/I/L)XGG-X in precursor proteins (Webster *et al*., 1989). Much of the knowledge about the requirement of proteolytic cleavage of some precursor proteins for maturation of adenovirus has come from the

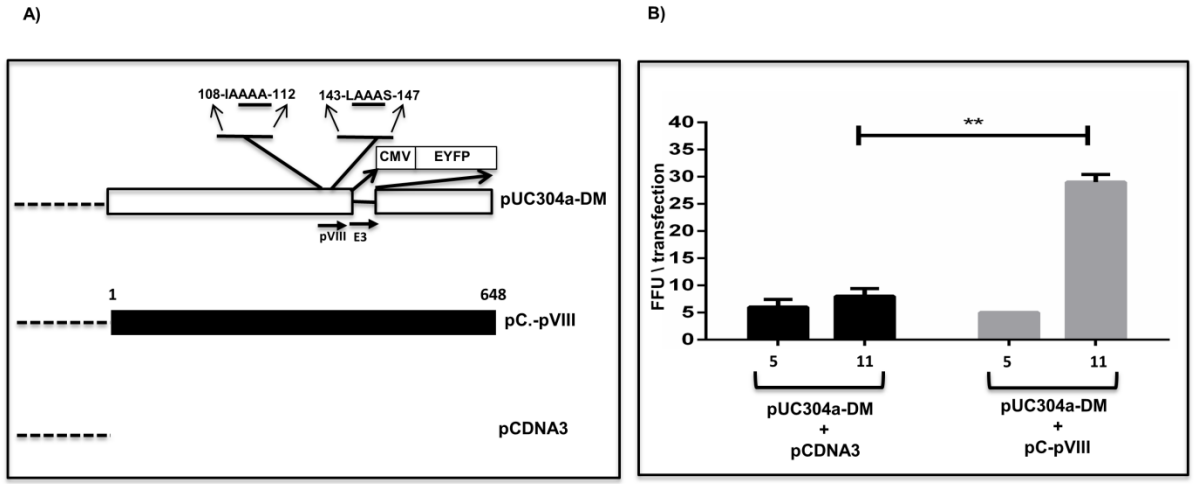


Figure 5.3.10 Complementation assay. (A). Schematic representation of plasmid pUC304a-DM. The dotted line represents the plasmid sequence. The BAdV-3 sequence is represented by hollow box. The thin line represents deleted E3 region (Zakhartchouk *et al*., 1998b). The alanines substituted for glycines are underlined. The numbers indicate the amino acids of BAdV-3 pVIII; CMV (human cytomegalo virus immediate early promoter); EYFP (enhanced yellow fluorescent protein). Arrows indicate the direction of the transcription. The black box represents the nucleotide sequence of BAdV-3 pVIII. The dotted line represents the nucleotide sequence of plasmid DNA. **(B).** Plasmid DNA from indicated plasmids was used to transfect the VIDO DT1 cells. At indicated days post transfection (denoted on X axis) fluorescent focus forming units were counted. Values represent averages from two independent repeats and error bars indicate the standard deviations. Statistical differences among the groups were calculated using unpaired t-test. Differences were considered significant at * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

studies involving a temperature sensitive mutant of HAdV-2 (Weber., 1976). However, the importance of cleavage of each precursor protein in determining the infectivity of progeny virus is not clear (Mangel & San Martín., 2014). A recent study has demonstrated the importance of N-terminal cleavage of pVI in cell entry and capsid assembly (Moyer *et al*., 2015). Thus, a better understanding of this complex process of proteolytic maturation of adenovirus needs further investigation. Here, we have analysed the role of cleavage of pVIII in BAdV-3 virus assembly, stability and infection. To our knowledge, this is the first study to investigate the role of proteolytic cleavage of pVIII in adenoviral life cycle.

Analysis of the BAdV-3 pVIII amino acid sequence revealed two potential protease cleavage sites (^{108}IAGG-G^{112}) (^{143}LGGG-S^{147}). Our results suggest that BAdV-3 protease cleaves BAdV-3 pVIII at both potential protease cleavage sites, which appears specific as mutation of protease cleavage sites abrogated the cleavage of pVIII. Moreover, cleavage at both potential sites does not appear to be sequential since mitigating cleavage at ^{108}IAGG-G^{112} motif does not block cleavage at ^{143}LGGG-S^{147} motif and vice versa. Earlier report has suggested the usage of only one potential protease cleavage site (^{143}LGGG-S^{147}) of pVIII (Ayalew *et al*., 2014). The difference could be due to use of different approach. Similarly, analysis of HAdV-5 pVIII revealed three potential adenoviral protease cleavage sites (amino acids 111, 131 and 157). Our results suggest that HAdV-5 protease can cleave pVIII at all three potential protease cleavage sites. Earlier studies have suggested the usage of only two potential cleavage sites at amino acid 111 and amino acid 157 of HAdV-5 pVIII (Blanche *et al*., 2001,Chelius *et al*., 2002,Lehmberg *et al*., 1999,Liu *et al*., 2003).

Analysis of the amino acid sequence revealed high degree of homology between proteases encoded by BAdV-3, HAdV-2 and PAdV-3 (Reddy *et al*., 1998). Moreover, the conservation of residues involved in the catalytic activity among proteases encoded by BAdV-3, HAdV-5 and PAdV-3 (Reddy *et al*., 1998) suggested that mechanism of cleavage of proteins harboring the potential cleavage site(s) might be conserved in *Mastadenoviruses.* Interestingly, detection of cleavage of pVIII encoded by BAdV-3, HAdV-5 or PAdV-3 by individual protease encoded by BAdV-3, HAdV-5 and PAdV-3 at potential protease cleavage sites provides evidence that the mechanism of cleavage of proteins appears conserved in members of *Mastadenoviruses.* However, we detected differences in the efficiency of usage of protease cleavage sites between HAdV-5, BAdV-3 and PAdV-3 protease. Earlier reports have suggested that efficiency of cleavage by adenovirus protease is dependent upon amino acid sequence at the cleavage site. The cleavage site sequence that conforms to GX-G type is cleaved more efficiently compared to cleavage site sequence that conforms to GG-X type (Diouri *et al*., 1996). Moreover, the four amino acids on either side of scissile bond determine the specificity of adenovirus protease mediated cleavage (Ruzindana-Umunyana *et al*., 2002,Webster *et al*., 1989). Our results confirm these findings as BAdV-3 pVIII was cleaved more efficiently at PPCS1 (GX-G type) than PPCS2 (GG-X type). Interestingly, there appears to be some difference in preference of cleavage site between HAdV-5, BAdV-3 and PAdV-3 protease as each of them cleaved different HAdV-5 pVIII cleavage sites with different efficiency.

Although abrogation of protease cleavage at either potential cleavage site (PPCS1 or PPCS2) affects the efficient production of infectious progeny virions, the protease cleavage at individual sites does not appear to be essential for the production of progeny BAdV-3. However, alteration of both protease cleavage sites (PPCS1 [amino acid 110-112] and PPCS2 [amino acids

146-148)] of BAdV-3 pVIII by substitution of glycines with alanines obviated the production of progeny virus suggesting that the absence of protease cleavage of pVIII is lethal for the production of progeny BAdV-3. These results are consistent with the suggestion that the function of the each protease cleavage site of BAdV-3 pVIII in viral replication may be redundant.

The cleavage of few precursor proteins including pVIII has been postulated to be required for the production of infectious progeny virions (Greber *et al*., 1996). Absence of cleavage of these precursor proteins appear to make virions non-infectious as it partially alter the uncoating and release of virions from the endosome (Cotten & Weber., 1995,Gastaldelli *et al*., 2008,Perez-Berna *et al*., 2012). Mutant viruses produced significantly less infectious particles than wild-type virus when cells were infected with equal number of infectious virus particles.

The defect could be in different steps of virus gene expression (receptor binding to virus assembly) including endosome escape. Several lines of evidence suggest that the capsid formation and virus assembly is occurring in mutant viruses. First, our quantitative TEM analysis suggests that the inability of cleavage at individual protease cleavage site of BAdV-3 pVIII does not appear to impair the release of partially uncoated BAdV-3 from the endosome(s). Second, there is no significant difference in the replication of mutant virus genomes. Third, alteration of individual protease cleavage site does not significantly affect the incorporation of proteins in mutant virus capsids. Finally, capsid formation and virus assembly appeared to occur as progeny virions produced in infected cells banded at CsCl gradient density, which is consistent with the formation of mature virions.

Earlier structural studies have revealed that mature HAdV-5 virion contain N- terminus (112 residues) and C-terminus (70 residues) proteolytic fragments of pVIII, which interact with the hexons (Reddy & Nemerow., 2014). While the ternary complex of V-VI-VIII stabilizes the

peripentonal hexons, the binary complex VIII- VIB contributes to the stabilization of group of nine hexons (GONs) (Reddy & Nemerow., 2014) providing help in maintaining the integrity of the capsids. Thus, significant decrease in the production of infectious BAdV-108A and BAdV-143A viruses could be due to the production of less stable virions. Indeed our results confirm the role of VIII in stabilization of virion capsid. First, the altered proteolytic processing of pVIII of BAdV-108A or BAdV-143A virus resulted in the production of thermolabile virions with fragile capsids, which may be disrupted by CsCl \TEM leading to the production of significant number of broken capsids. Secondly, cleavage of pVIII at both potential cleavage sites appears essential for maintaining the integrity and stability of BAdV-3 virion.

In conclusion, we have demonstrated that mitigation of both protease cleavage sites of pVIII is detrimental for progeny virus production. Moreover, single pVIII protease cleavage site mutants assemble virus particles. However, these thermolabile virus particles may lead to the production of non-infectious virions with broken capsids.

6.0 GENERAL DISCUSSION AND CONCLUSION

To understand the basic biology of BAdV-3 it is necessary to study the biological functions of the various structural and non-structural viral proteins. This study is continuation of our earlier work on characterizing one of the structural protein pVIII of BAdV-3 which is also one of the least characterized adenovirus proteins.

Protein-protein interactions influence every cellular process. Similarly, the interaction of viral proteins and host cell proteins is essential for virus replication, growth and survival. Earlier, we demonstrated that BAdV-3 pVIII a) is expressed as 24 kDa protein in BAdV-3 infected or gene transfected cells and is localized both in the nucleus and the cytoplasm, b) N-terminal region (amino acids 57-72) of pVIII is involved in nuclear transport of pVIII by interacting preferentially with importin α-3 (Ayalew *et al*., 2014), and c) interacts with cellular factor DDX3, which alters the cap dependent translation by excluding eIF's from cap binding complex (Ayalew., 2015).

Using yeast two-hybrid analysis, we observed that pVIII may also interact with cellular protein eIF6. This pVIII-eIF6 interaction was confirmed *in-vitro* and *in-vivo* using GST pull-down, BiFC and co-immunoprecipitation assays.

The eIF6 is a 245 amino acid long protein that is highly conserved (Si *et al*., 1997,Si & Maitra., 1999). eIF6 has a dual role and is important both for ribosome biogenesis and protein translation. It is associated with 60S subunit, which helps to prevent joining of 40S subunit and immature 60S subunit. After maturation of 60S subunit, eIF6 is released from the 60S subunit, which allows joining of 60S and 40S subunits leading to the formation of functional 80S ribosome (Gartmann *et al*., 2010,Russell & Spremulli., 1979,Valenzuela *et al*., 1982).

Using polysome profile analysis of BAdV-3 infected cells and pVIII expressing cell line we demonstrate that the presence of pVIII impairs joining of 40S and 60S subunits which consequently leads to reduction in formation of functional 80S subunits. We speculate that this impairment of formation of functional 80S unit may be because of inhibition of release of eIF6 from mature 60S subunit, thus altering the cellular mRNA synthesis.

Viruses do not have their own metabolic machinery so they depend completely on their host for translation of their transcripts. Many viruses globally interfere with the host translation by inhibiting cap dependent ribosome recruitment to host mRNA (Firth & Brierley., 2012,Gale *et al*., 2000). Earlier studies have reported inhibition of cellular protein synthesis during adenovirus infection particularly during late times post infection (Dolph *et al*., 1988,Huang & Schneider., 1991) indicating that one or more late adenoviral proteins inhibit cap dependent translation of cellular mRNAs. Earlier studies have implicated 100K protein for inhibiting cellular protein synthesis (Cuesta *et al*., 2004). However, it is possible that viruses may employ more than one mechanism to control the complex process of translation and hence more than one viral protein may be involved in inhibiting cellular protein synthesis.

It is possible that interaction of pVIII and eIF6 may have a role in preferential translation of late viral mRNAs. Support for this comes from the fact that abrogating the interaction of pVIII-eIF6 results in decreased production of progeny virus BAdV-3-d147-174 because of significant decrease in the production of late viral proteins. It is possible that interaction of pVIII-eIF6 allows availability of free 40S and 60Sribosome subunits, which may allow the recruitment of free 40S and 60S ribosomal units to late viral mRNA that contain tripartite leader sequence (TPL) leading to their preferential translation. Alternatively, it is possible that inhibition of cellular mRNA translation at late times post infection by different mechanisms

involving BAdV-3 100K (Cuesta *et al*., 2004) and pVIII may indirectly help the translation of late viral mRNAs by independent mechanism (Yueh & Schneider., 1996). However, the exact mechanism by which pVIII-eIF6 interaction modulates late viral protein synthesis remains to be determined.

The second part of the thesis determined the role of proteolytic cleavage in the maturation of progeny infectious BAdV-3. The maturation of immature adenovirus involving proteolytic processing of precursor proteins by adenovirus protease is an important step in adenovirus life cycle (Mangel & San Martín., 2014,Moyer *et al*., 2015). However, this complex process is still not fully understood especially how these individual proteolytic events affect adenovirus assembly stability and infectivity is not known.

Based on our experimental approach, earlier we reported that BAdV-3 pVIII is only cleaved at its C-terminal potential cleavage site ^{143}LGGG↓S^{147} (Ayalew *et al*., 2014). However, using a different approach here we demonstrated that BAdV-3 pVIII is cleaved at its both potential adenovirus protease cleavage sites. Further our results indicate that usage of at least one cleavage site is essential for the production of progeny BAdV-3 virions as glycine to alanine mutation of both protease cleavage sites appears lethal for the production of progeny virions. Interestingly, cleavage of pVIII at both potential cleavage sites appears essential for the production of stable BAdV-3 virions as BAdV-3 expressing pVIII containing glycine to alanine mutation of either of the potential cleavage site are thermolabile and may lead to production of non- infectious virions with disrupted capsids.

Earlier structural studies have suggested that pVIII stabilize peripentonal and group of nine hexons (GONs) (Reddy & Nemerow., 2014). Indeed, our results confirm these findings as mutation of individual cleavage site of pVIII resulted in production of thermolabile and fragile

virions. It is possible that mutation of individual cleavage site of pVIII resulted in generation of pVIII fragments that does not fit well in the virion structure and hence can no longer interact and stabilize hexons resulting in production of fragile and increased number of non-infectious particles. Alternatively, it is possible that alteration of cleavage sites results in generation of pVIII fragments that fold differently and are unable to form complexes with other structural proteins thereby affecting the stability of the virus particle.

The results of characterization of BAdV-3 pVIII carried out in this study provide evidence that pVIII is an important protein in adenoviral life cycle. The pVIII-eIF6 interaction inhibits cellular protein synthesis and diverts the cellular resources for preferential translation of late adenovirus mRNAs. Moreover, we provide evidence that cleavage of pVIII at atleast one potential cleavage sites appears essential for the formation and production of mature virions. To our knowledge, this is the first study to investigate the role of proteolytic cleavage of pVIII in adenoviral life cycle.

7.0 FUTURE DIRECTIONS

In the present study, we characterized interaction of BAdV-3 pVIII with cellular protein eIF6. Our polysome profile results indicate that in the presence of BAdV-3 pVIII the formation of functional 80S ribosome is inhibited. We speculate that this might be because of inhibition of release of eIF6 from 60S subunit. So future work could focus on determining the mechanism by which pVIII inhibit release of eIF6 from 60S subunit.

Secondly, our results of polysome profile indicate that free 40S subunits are diminished significantly in BAdV-3 infected cells compared to free 40S subunits in mock infected cells suggesting that some adenoviral protein(s) affects 40S ribosome biogenesis. So experiments can be performed to determine the adenoviral protein that affects the 40S ribosome biogenesis.

Thirdly, the fate of cleaved middle fragment (amino acid 112 to 146) of BAdV-3 pVIII is still not known. A recombinant virus can be made with a tag inserted just after the first protease cleavage site of BAdV-3 pVIII or just before the second cleavage site of BAdV-3 pVIII and experiments can be done with this recombinant virus to determine whether the cleaved middle fragment of BAdV-3 pVIII is incorporated in the mature virus particle or not. Alternatively, an antibody can be generated that specifically detects the cleaved middle fragment (amino acid 112 to 146) and can be used to detect presence or absence of amino acid 112 to 146 of pVIII in mature virus.

8.0 REFRENCES

Ahi Y. S., Vemula S. V., Mittal S. K. (2013). Adenoviral E2 IVa2 protein interacts with L4 33K protein and E2 DNA-binding protein. *J Gen Virol* **94**, 1325-1334.

Ahi Y. S., Vemula S. V., Hassan A. O., Costakes G., Stauffacher C., Mittal S. K. (2015). Adenoviral L4 33K forms ring-like oligomers and stimulates ATPase activity of IVa2: Implications in viral genome packaging. *Frontiers in Microbiology* **6**.

Akalu A., Liebermann H., Bauer U., Granzow H., Seidel W. (1999). The subgenus-specific C-terminal region of protein IX is located on the surface of the adenovirus capsid. *J Virol* **73**, 6182-6187.

Amin M., Mirza A., Weber J. (1977). Genetic analysis of adenovirus type 2: VII. cleavage-modified affinity for DNA of internal virion proteins. *Virology* **80**, 83-97.

Amrani N., Ghosh S., Mangus D. A., Jacobson A. (2008). Translation factors promote the formation of two states of the closed-loop mRNP. *Nature* **453**, 1276-1280.

Anand S. K., Gaba A., Singh J., Tikoo S. K. (2014). Bovine adenovirus 3 core protein precursor pVII localizes to mitochondria, and modulates ATP synthesis, mitochondrial Ca2+ and mitochondrial membrane potential. *J Gen Virol* **95**, 442-452.

Anderson C. W. (1990). The proteinase polypeptide of adenovirus serotype 2 virions. *Virology* **177**, 259-272.

Anderson C. W., Young M. E., Flint S. (1989). Characterization of the adenovirus 2 virion protein, mu. *Virology* **172**, 506-512.

Anderson C. W., Baum P. R., Gesteland R. F. (1973). Processing of adenovirus 2-induced proteins. *J Virol* **12**, 241-252.

Andersson M. G., Haasnoot P. C., Xu N., Berenjian S., Berkhout B., Akusjarvi G. (2005). Suppression of RNA interference by adenovirus virus-associated RNA. *J Virol* **79**, 9556-9565.

Aoki K. & Tagawa Y. (2002). A twenty-one year surveillance of adenoviral conjunctivitis in sapporo, japan. *Int Ophthalmol Clin* **42**, 49-54.

Aoyagi M., Gaspar M., Shenk T. E. (2010). Human cytomegalovirus UL69 protein facilitates translation by associating with the mRNA cap-binding complex and excluding 4EBP1. *Proc Natl Acad Sci U S A* **107**, 2640-2645.

Arnberg N. (2012). Adenovirus receptors: Implications for targeting of viral vectors. *Trends Pharmacol Sci* **33**, 442-448.

Arnberg N. (2009). Adenovirus receptors: Implications for tropism, treatment and targeting. *Rev Med Virol* **19**, 165-178.

Arnberg N., Kidd A. H., Edlund K., Olfat F., Wadell G. (2000). Initial interactions of subgenus D adenoviruses with A549 cellular receptors: Sialic acid versus alpha(v) integrins. *J Virol* **74**, 7691-7693.

Atkinson R., Dhurandhar N., Allison D., Bowen R., Israel B., Albu J., Augustus A. (2005). Human adenovirus-36 is associated with increased body weight and paradoxical reduction of serum lipids. *Int J Obes* **29**, 281-286.

Avvakumov N., Kajon A., Hoeben R., Mymryk J. (2004). Comprehensive sequence analysis of the E1A proteins of human and simian adenoviruses. *Virology* **329**, 477-492.

Ayalew L. (2015). The role of bovine adenovirus (BAdV)-3 protein pVIII in virus replication. PhD thesis. University of Saskatchewan, Saskatoon, Sk, Canada.

Ayalew L. E., Gaba A., Kumar P., Tikoo S. K. (2014). Conserved regions of bovine adenovirus-3 pVIII contain functional domains involved in nuclear localization and packaging in mature infectious virions. *J Gen Virol* **95**, 1743-1754.

Backström E., Kaufmann K. B., Lan X., Akusjärvi G. (2010). Adenovirus L4-22K stimulates major late transcription by a mechanism requiring the intragenic late-specific transcription factor-binding site. *Virus Res* **151**, 220-228.

Bai M., Harfe B., Freimuth P. (1993). Mutations that alter an arg-gly-asp (RGD) sequence in the adenovirus type 2 penton base protein abolish its cell-rounding activity and delay virus reproduction in flat cells. *J Virol* **67**, 5198-5205.

Bajpayee N. S., McGrath W. J., Mangel W. F. (2005). Interaction of the adenovirus proteinase with protein cofactors with high negative charge densities. *Biochemistry (N Y)* **44**, 8721-8729.

Baldwin A., Kingman H., Darville M., Foot A., Grier D., Cornish J., Goulden N., Oakhill A., Pamphilon D., Steward C. (2000). Outcome and clinical course of 100 patients with adenovirus infection following bone marrow transplantation. *Bone Marrow Transplant* **26**, 1333-1338.

Baniecki M. L., McGrath W. J., McWhirter S. M., Li C., Toledo D. L., Pellicena P., Barnard D. L., Thorn K. S., Mangel W. F. (2001). Interaction of the human adenovirus proteinase with its 11-amino acid cofactor pVIc. *Biochemistry (N Y)* **40**, 12349-12356.

Basu U., Si K., Deng H., Maitra U. (2003). Phosphorylation of mammalian eukaryotic translation initiation factor 6 and its saccharomyces cerevisiae homologue Tif6p: Evidence that phosphorylation of Tif6p regulates its nucleocytoplasmic distribution and is required for yeast cell growth. *Mol Cell Biol* **23**, 6187-6199.

Basu U., Si K., Warner J. R., Maitra U. (2001). The saccharomyces cerevisiae TIF6 gene encoding translation initiation factor 6 is required for 60S ribosomal subunit biogenesis. *Mol Cell Biol* **21**, 1453-1462.

Baxi M. K., Robertson J., Babiuk L. A., Tikoo S. K. (2001). Mutational analysis of early region 4 of bovine adenovirus type 3. *Virology* **290**, 153-163.

Baxi M. K., Babiuk L. A., Mehtali M., Tikoo S. K. (1999). Transcription map and expression of bovine herpesvirus-1 glycoprotein D in early region 4 of bovine adenovirus-3. *Virology* **261**, 143-152.

Baxi M. K., Reddy P. S., Zakhartchouk A. N., Idamakanti N., Pyne C., Babiuk L. A., Tikoo S. K. (1998). Characterization of bovine adenovirus type 3 early region 2B. *Virus Genes* **16**, 313-316.

Bécam A., Nasr F., Racki W., Zagulski M., Herbert C. (2001). Ria1p (Ynl163c), a protein similar to elongation factors 2, is involved in the biogenesis of the 60S subunit of the ribosome in saccharomyces cerevisiae. *Molecular Genetics and Genomics* **266**, 454-462.

Benkö M. & Harrach B. (1998). A proposal for a new (third) genus within the family adenoviridae. *Arch Virol* **143**, 829-837.

Benkö M., Bartha A., Wadell G. (1988). DNA restriction enzyme analysis of bovine adenoviruses. *Intervirology* **29**, 346-350.

Bennasser Y., Chable-Bessia C., Triboulet R., Gibbings D., Gwizdek C., Dargemont C., Kremer E. J., Voinnet O., Benkirane M. (2011). Competition for XPO5 binding between dicer mRNA, pre-miRNA and viral RNA regulates human dicer levels. *Nature Structural & Molecular Biology* **18**, 323-327.

Bennett E. M., Bennink J. R., Yewdell J. W., Brodsky F. M. (1999). Cutting edge: Adenovirus E19 has two mechanisms for affecting class I MHC expression. *J Immunol* **162**, 5049-5052.

Bergelson J. M., Cunningham J. A., Droguett G., Kurt-Jones E. A., Krithivas A., Hong J. S., Horwitz M. S., Crowell R. L., Finberg R. W. (1997). Isolation of a common receptor for coxsackie B viruses and adenoviruses 2 and 5. *Science* **275**, 1320-1323.

Berget S. M., Moore C., Sharp P. A. (1977). Spliced segments at the 5' terminus of adenovirus 2 late mRNA. *Proc Natl Acad Sci U S A* **74**, 3171-3175.

Berk A. (2013). Adenoviridae. *Field's Virology, 6th Edn.Lippincott Williams & Wilkins, Philadelphia* , 1704-1731.

Berk A. J. (2005). Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus. *Oncogene* **24**, 7673-7685.

Berk A. J. & Sharp P. A. (1978). Structure of the adenovirus 2 early mRNAs. *Cell* **14**, 695-711.

Bhatti A. R. & Weber J. (1979). Protease of adenovirus type 2: Partial characterization. *Virology* **96**, 478-485.

Bhatti A. & Weber J. (1978). Protease of adenovirus type 2. in vitro processing of core protein. *Biochem Biophys Res Commun* **81**, 973-979.

Biffo S., Sanvito F., Costa S., Preve L., Pignatelli R., Spinardi L., Marchisio P. C. (1997). Isolation of a novel β4 integrin-binding protein (p27BBP) highly expressed in epithelial cells. *J Biol Chem* **272**, 30314-30321.

Bines J. E., Liem N. T., Justice F. A., Son T. N., Kirkwood C. D., De Campo M., Barnett P., Bishop R. F., Robins-Browne R., Carlin J. B. (2006). Risk factors for intussusception in infants in vietnam and australia: Adenovirus implicated, but not rotavirus. *J Pediatr* **149**, 452-460. e1.

Biswas A., Choudhuri A., Maitra U. (2012). TIF6 (eIF6). In *Encyclopedia of Signaling Molecules*pp. 1859-1866. Edited by Anonymous : Springer.

Biswas A., Mukherjee S., Das S., Shields D., Chow C. W., Maitra U. (2011). Opposing action of casein kinase 1 and calcineurin in nucleo-cytoplasmic shuttling of mammalian translation initiation factor eIF6. *J Biol Chem* **286**, 3129-3138.

Blakqori G., van Knippenberg I., Elliott R. M. (2009). Bunyamwera orthobunyavirus S-segment untranslated regions mediate poly(A) tail-independent translation. *J Virol* **83**, 3637-3646.

Blanche F., Monegier B., Faucher D., Duchesne M., Audhuy F., Barbot A., Bouvier S., Daude G., Dubois H., Guillemin T. (2001). Polypeptide composition of an adenovirus type 5 used in cancer gene therapy. *Journal of Chromatography A* **921**, 39-48.

Bosher J., Robinson E. C., Hay R. T. (1990). Interactions between the adenovirus type 2 DNA polymerase and the DNA binding domain of nuclear factor I. *New Biol* **2**, 1083-1090.

Bowles N. E., Ni J., Kearney D. L., Pauschinger M., Schultheiss H., McCarthy R., Hare J., Bricker J. T., Bowles K. R., Towbin J. A. (2003). Detection of viruses in myocardial tissues by polymerase chain reaction: Evidence of adenovirus as a common cause of myocarditis in children and adults. *J Am Coll Cardiol* **42**, 466-472.

Boyer J. L. & Ketner G. (2000). Genetic analysis of a potential zinc-binding domain of the adenovirus E4 34k protein. *J Biol Chem* **275**, 14969-14978.

Bremner K. H., Scherer J., Yi J., Vershinin M., Gross S. P., Vallee R. B. (2009). Adenovirus transport via direct interaction of cytoplasmic dynein with the viral capsid hexon subunit. *Cell Host & Microbe* **6**, 523-535.

Bridges N. D., Spray T. L., Collins M. H., Bowles N. E., Towbin J. A. (1998). Adenovirus infection in the lung results in graft failure after lung transplantation. *J Thorac Cardiovasc Surg* **116**, 617-623.

Brown J., Fauquet C., Briddon R., Zerbini M., Moriones E., Navas-Castillo J., King A., Adams M., Carstens E., Lefkowitz E. (2012). Virus taxonomy: Ninth report of the international committee on taxonomy of viruses. *King, AMQ, Lefkowitz, E., Adams, MJ, Carstens, EB, Eds* , 351373.

Brown M. T. & Mangel W. F. (2004). Interaction of actin and its 11-amino acid C-terminal peptide as cofactors with the adenovirus proteinase. *FEBS Lett* **563**, 213-218.

Brown M. T., McGrath W. J., Toledo D. L., Mangel W. F. (1996). Different modes of inhibition of human adenovirus proteinase, probably a cysteine proteinase, by bovine pancreatic trypsin inhibitor. *FEBS Lett* **388**, 233-237.

Brown M. T., McBride K. M., Baniecki M. L., Reich N. C., Marriott G., Mangel W. F. (2002). Actin can act as a cofactor for a viral proteinase in the cleavage of the cytoskeleton. *J Biol Chem* **277**, 46298-46303.

Burgert H. G., Maryanski J. L., Kvist S. (1987). "E3/19K" protein of adenovirus type 2 inhibits lysis of cytolytic T lymphocytes by blocking cell-surface expression of histocompatibility class I antigens. *Proc Natl Acad Sci U S A* **84**, 1356-1360.

Burgui I., Yanguez E., Sonenberg N., Nieto A. (2007). Influenza virus mRNA translation revisited: Is the eIF4E cap-binding factor required for viral mRNA translation? *J Virol* **81**, 12427-12438.

Bushell M. & Sarnow P. (2002). Hijacking the translation apparatus by RNA viruses. *J Cell Biol* **158**, 395-399.

Byrd M. P., Zamora M., Lloyd R. E. (2005). Translation of eukaryotic translation initiation factor 4GI (eIF4GI) proceeds from multiple mRNAs containing a novel cap-dependent internal ribosome entry site (IRES) that is active during poliovirus infection. *J Biol Chem* **280**, 18610-18622.

Cai F. X., Bourbonniere M., Tang D., Hu S. L., Weber J. M. (1990). Nucleotide and deduced amino acid sequence of the bovine adenovirus type 3 proteinase. *Nucleic Acids Res* **18**, 5568.

Caillet-Boudin M. (1989). Complementary peptide sequences in partner proteins of the adenovirus capsid. *J Mol Biol* **208**, 195-198.

Cames B., Rahier J., Burtomboy G., de Goyet, Jean de Ville, Reding R., Lamy M., Otte J. B., Sokal E. M. (1992). Acute adenovirus hepatitis in liver transplant recipients. *J Pediatr* **120**, 33-37.

Campos S. K. & Barry M. A. (2007). Current advances and future challenges in adenoviral vector biology and targeting. *Curr Gene Ther* **7**, 189-204.

Cassany A., Ragues J., Guan T., Begu D., Wodrich H., Kann M., Nemerow G. R., Gerace L. (2015). Nuclear import of adenovirus DNA involves direct interaction of hexon with an N-terminal domain of the nucleoporin Nup214. *J Virol* **89**, 1719-1730.

Castelló A., Quintas A., Sánchez E. G., Sabina P., Nogal M., Carrasco L., Revilla Y. (2009). Regulation of host translational machinery by african swine fever virus. *PLoS Pathog* **5**, e1000562.

Ceci M., Gaviraghi C., Gorrini C., Sala L. A., Offenhauser N., Marchisio P. C., Biffo S. (2003). Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly. *Nature* **426**, 579-584.

Cepko C. L. & Sharp P. A. (1982). Assembly of adenovirus major capsid protein is mediated by a nonvirion protein. *Cell* **31**, 407-415.

Chakravarti D., Ogryzko V., Kao H., Nash A., Chen H., Nakatani Y., Evans R. M. (1999). A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. *Cell* **96**, 393-403.

Challberg M. D. & Kelly T. J.,Jr. (1981). Processing of the adenovirus terminal protein. *J Virol* **38**, 272-277.

Challberg M. D., Desiderio S. V., Kelly T. J.,Jr. (1980). Adenovirus DNA replication in vitro: Characterization of a protein covalently linked to nascent DNA strands. *Proc Natl Acad Sci U S A* **77**, 5105-5109.

Chatterjee P. K., Vayda M. E., Flint S. (1986). Identification of proteins and protein domains that contact DNA within adenovirus nucleoprotein cores by ultraviolet light crosslinking of oligonucleotides 32 P-labelled in vivo. *J Mol Biol* **188**, 23-37.

Chattopadhyay D., Ghosh M. K., Mal A., Harter M. L. (2001). Inactivation of p21 by E1A leads to the induction of apoptosis in DNA-damaged cells. *J Virol* **75**, 9844-9856.

Chelius D., Hühmer A. F., Shieh C. H., Lehmberg E., Traina J. A., Slattery T. K., Pungor E. (2002). Analysis of the adenovirus type 5 proteome by liquid chromatography and tandem mass spectrometry methods. *Journal of Proteome Research* **1**, 501-513.

Chen H., Vinnakota R., Flint S. J. (1994). Intragenic activating and repressing elements control transcription from the adenovirus IVa2 initiator. *Mol Cell Biol* **14**, 676-685.

Chen M., Mermod N., Horwitz M. S. (1990). Protein-protein interactions between adenovirus DNA polymerase and nuclear factor I mediate formation of the DNA replication preinitiation complex. *J Biol Chem* **265**, 18634-18642.

Chen P. H., Ornelles D. A., Shenk T. (1993). The adenovirus L3 23-kilodalton proteinase cleaves the amino-terminal head domain from cytokeratin 18 and disrupts the cytokeratin network of HeLa cells. *J Virol* **67**, 3507-3514.

Chin Y. R. & Horwitz M. S. (2006). Adenovirus RID complex enhances degradation of internalized tumour necrosis factor receptor 1 without affecting its rate of endocytosis. *J Gen Virol* **87**, 3161-3167.

Chiocca S., Kurzbauer R., Schaffner G., Baker A., Mautner V., Cotten M. (1996). The complete DNA sequence and genomic organization of the avian adenovirus CELO. *J Virol* **70**, 2939-2949.

Chou S., Roos R., Burrell R., Gutmann L., Harley J. (1973). Subacute focal adenovirus encephalitis. *Journal of Neuropathology & Experimental Neurology* **32**, 34-50.

Chow L. T., Gelinas R. E., Broker T. R., Roberts R. J. (1977). An amazing sequence arrangement at the 5′ ends of adenovirus 2 messenger RNA. *Cell* **12**, 1-8.

Christensen J. B., Byrd S. A., Walker A. K., Strahler J. R., Andrews P. C., Imperiale M. J. (2008). Presence of the adenovirus IVa2 protein at a single vertex of the mature virion. *J Virol* **82**, 9086-9093.

Chroboczek J., Ruigrok R., Cusack S. (1995). Adenovirus fiber. In *The Molecular Repertoire of Adenoviruses I*pp. 163-200. Edited by Anonymous : Springer.

Chroboczek J., Bieber F., Jacrot B. (1992). The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2. *Virology* **186**, 280-285.

Chung S. H., Frese K. K., Weiss R. S., Prasad B. V., Javier R. T. (2007). A new crucial protein interaction element that targets the adenovirus E4-ORF1 oncoprotein to membrane vesicles. *J Virol* **81**, 4787-4797.

Clyde K. & Glaunsinger B. A. (2010). 1 getting the message: Direct manipulation of host mRNA accumulation during gammaherpesvirus lytic infection. *Adv Virus Res* **78**, 1.

Condezo G. N., Marabini R., Ayora S., Carazo J. M., Alba R., Chillon M., San Martin C. (2015). Structures of adenovirus incomplete particles clarify capsid architecture and show maturation changes of packaging protein L1 52/55k. *J Virol* **89**, 9653-9664.

Connor J. H. & Lyles D. S. (2002). Vesicular stomatitis virus infection alters the eIF4F translation initiation complex and causes dephosphorylation of the eIF4E binding protein 4E-BP1. *J Virol* **76**, 10177-10187.

Cotten M. & Weber J. M. (1995). The adenovirus protease is required for virus entry into host cells. *Virology* **213**, 494-502.

Coyne C. B. & Bergelson J. M. (2005). CAR: A virus receptor within the tight junction. *Adv Drug Deliv Rev* **57**, 869-882.

Cuesta R., Xi Q., Schneider R. J. (2004). Structural basis for competitive inhibition of eIF4G-Mnk1 interaction by the adenovirus 100-kilodalton protein. *J Virol* **78**, 7707-7716.

Cuesta R., Xi Q., Schneider R. J. (2000). Adenovirus-specific translation by displacement of kinase Mnk1 from cap-initiation complex eIF4F. *Embo J* **19**, 3465-3474.

Darbyshire J. H., Dawson P. S., Lamont P. H., Ostler D. C., Pereira H. G. (1965). A new adenovirus serotype of bovine origin. *J Comp Pathol* **75**, 327-330.

Davison A. J., Benkő M., Harrach B. (2003). Genetic content and evolution of adenoviruses. *J Gen Virol* **84**, 2895-2908.

Debbas M. & White E. (1993). Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B. *Genes Dev* **7**, 546-554.

Dekker J., Kanellopoulos P. N., Loonstra A. K., van Oosterhout J. A., Leonard K., Tucker P. A., van der Vliet P. C. (1997). Multimerization of the adenovirus DNA-binding protein is the driving force for ATP-independent DNA unwinding during strand displacement synthesis. *Embo J* **16**, 1455-1463.

Dennert R., Crijns H. J., Heymans S. (2008). Acute viral myocarditis. *Eur Heart J* **29**, 2073-2082.

Ding J., McGrath W. J., Sweet R. M., Mangel W. F. (1996). Crystal structure of the human adenovirus proteinase with its 11 amino acid cofactor. *Embo J* **15**, 1778-1783.

Diouri M., Keyvani-Amineh H., Geoghegan K. F., Weber J. M. (1996). Cleavage efficiency by adenovirus protease is site-dependent. *J Biol Chem* **271**, 32511-32514.

Dmitriev I. P., Kashentseva E. A., Curiel D. T. (2002). Engineering of adenovirus vectors containing heterologous peptide sequences in the C terminus of capsid protein IX. *J Virol* **76**, 6893-6899.

Dobner T., Horikoshi N., Rubenwolf S., Shenk T. (1996). Blockage by adenovirus E4orf6 of transcriptional activation by the p53 tumor suppressor. *Science* **272**, 1470-1473.

Dolph P. J., Racaniello V., Villamarin A., Palladino F., Schneider R. J. (1988). The adenovirus tripartite leader may eliminate the requirement for cap-binding protein complex during translation initiation. *J Virol* **62**, 2059-2066.

Dominski Z. & Marzluff W. F. (2007). Formation of the 3′ end of histone mRNA: Getting closer to the end. *Gene* **396**, 373-390.

Donadini A., Giodini A., Sanvito F., Marchisio P. C., Biffo S. (2001). The human ITGB4BP gene is constitutively expressed in vitro, but highly modulated in vivo. *Gene* **266**, 35-43.

Dou S., Zeng X., Cortes P., Erdjument-Bromage H., Tempst P., Honjo T., Vales L. D. (1994). The recombination signal sequence-binding protein RBP-2N functions as a transcriptional repressor. *Mol Cell Biol* **14**, 3310-3319.

Du E. & Tikoo S. K. (2010). Efficient replication and generation of recombinant bovine adenovirus-3 in nonbovine cotton rat lung cells expressing I-SceI endonuclease. *J Gene Med* **12**, 840-847.

Dubberke E. R., Tu B., Rivet D. J., Storch G. A., Apisarnthanarak A., Schmidt R. E., Weiss S., Polish L. B. (2006). Acute meningoencephalitis caused by adenovirus serotype 26. *J Neurovirol* **12**, 235-240.

Dunn E. F. & Connor J. H. (2011). Dominant inhibition of akt/protein kinase B signaling by the matrix protein of a negative-strand RNA virus. *J Virol* **85**, 422-431.

Echavarria M. (2009). Adenoviruses. In *Principles and Practice of Clinical Virology*, 6th edn, pp. 1014. Edited by A. J. Zuckerman. Chichester, UK ; Hoboken, NJ: John Wiley & Sons.

Edvardsson B., Everitt E., Jornvall H., Prage L., Philipson L. (1976). Intermediates in adenovirus assembly. *J Virol* **19**, 533-547.

ÉlŐ P., Farkas S. L., Dán Á L., Kovacs G. M. (2003). The p32K structural protein of the atadenovirus might have bacterial relatives. *J Mol Evol* **56**, 175-180.

Erickson F. L. & Hannig E. M. (1996). Ligand interactions with eukaryotic translation initiation factor 2: Role of the gamma-subunit. *Embo J* **15**, 6311-6320.

Etchison D., Milburn S. C., Edery I., Sonenberg N., Hershey J. W. (1982). Inhibition of HeLa cell protein synthesis following poliovirus infection correlates with the proteolysis of a 220,000-dalton polypeptide associated with eucaryotic initiation factor 3 and a cap binding protein complex. *J Biol Chem* **257**, 14806-14810.

Ewing S. G., Byrd S. A., Christensen J. B., Tyler R. E., Imperiale M. J. (2007). Ternary complex formation on the adenovirus packaging sequence by the IVa2 and L4 22-kilodalton proteins. *J Virol* **81**, 12450-12457.

Feigenblum D. & Schneider R. J. (1993). Modification of eukaryotic initiation factor 4F during infection by influenza virus. *J Virol* **67**, 3027-3035.

Finch A. J., Hilcenko C., Basse N., Drynan L. F., Goyenechea B., Menne T. F., Gonzalez Fernandez A., Simpson P., D'Santos C. S.& other authors. (2011). Uncoupling of GTP hydrolysis from eIF6 release on the ribosome causes shwachman-diamond syndrome. *Genes Dev* **25**, 917-929.

Firth A. E. & Brierley I. (2012). Non-canonical translation in RNA viruses. *J Gen Virol* **93**, 1385-1409.

Fraser N. W., Baker C. C., Moore M. A., Ziff E. B. (1982). Poly (A) sites of adenovirus serotype 2 transcription units. *J Mol Biol* **155**, 207-233.

Fredman J. N. & Engler J. A. (1993). Adenovirus precursor to terminal protein interacts with the nuclear matrix in vivo and in vitro. *J Virol* **67**, 3384-3395.

Frese K. K., Lee S. S., Thomas D. L., Latorre I. J., Weiss R. S., Glaunsinger B. A., Javier R. T. (2003). Selective PDZ protein-dependent stimulation of phosphatidylinositol 3-kinase by the adenovirus E4-ORF1 oncoprotein. *Oncogene* **22**, 710-721.

Fütterer J., Kiss-László Z., Hohn T. (1993). Nonlinear ribosome migration on cauliflower mosaic virus 35S RNA. *Cell* **73**, 789-802.

Gale M.,Jr, Tan S. L., Katze M. G. (2000). Translational control of viral gene expression in eukaryotes. *Microbiol Mol Biol Rev* **64**, 239-280.

Gallimore P. H. & Turnell A. S. (2001). Adenovirus E1A: Remodelling the host cell, a life or death experience. *Oncogene* **20**.

Garrey J. L., Lee Y. Y., Au H. H., Bushell M., Jan E. (2010). Host and viral translational mechanisms during cricket paralysis virus infection. *J Virol* **84**, 1124-1138.

Gartmann M., Blau M., Armache J. P., Mielke T., Topf M., Beckmann R. (2010). Mechanism of eIF6-mediated inhibition of ribosomal subunit joining. *J Biol Chem* **285**, 14848-14851.

Gastaldelli M., Imelli N., Boucke K., Amstutz B., Meier O., Greber U. F. (2008). Infectious adenovirus type 2 transport through early but not late endosomes. *Traffic* **9**, 2265-2278.

Ghebremedhin B. (2014). Human adenovirus: Viral pathogen with increasing importance. *European Journal of Microbiology and Immunology* **4**, 26-33.

Gietz D., St Jean A., Woods R. A., Schiestl R. H. (1992). Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res* **20**, 1425.

Gingras A., Raught B., Sonenberg N. (1999). eIF4 initiation factors: Effectors of mRNA recruitment to ribosomes and regulators of translation. *Annu Rev Biochem* **68**, 913-963.

Gingras A. C., Raught B., Sonenberg N. (2001a). Regulation of translation initiation by FRAP/mTOR. *Genes Dev* **15**, 807-826.

Gingras A. C., Svitkin Y., Belsham G. J., Pause A., Sonenberg N. (1996). Activation of the translational suppressor 4E-BP1 following infection with encephalomyocarditis virus and poliovirus. *Proc Natl Acad Sci U S A* **93**, 5578-5583.

Gingras A. C., Raught B., Gygi S. P., Niedzwiecka A., Miron M., Burley S. K., Polakiewicz R. D., Wyslouch-Cieszynska A., Aebersold R., Sonenberg N. (2001b). Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev* **15**, 2852-2864.

Gorgoni B. & Gray N. K. (2004). The roles of cytoplasmic poly(A)-binding proteins in regulating gene expression: A developmental perspective. *Brief Funct Genomic Proteomic* **3**, 125-141.

Gorman J. J., Wallis T. P., Whelan D. A., Shaw J., Both G. W. (2005). LH3, a "homologue" of the mastadenoviral E1B 55-kDa protein is a structural protein of atadenoviruses. *Virology* **342**, 159-166.

Gray G. C., McCarthy T., Lebeck M. G., Schnurr D. P., Russell K. L., Kajon A. E., Landry M. L., Leland D. S., Storch G. A.& other authors. (2007). Genotype prevalence and risk factors for severe clinical adenovirus infection, united states 2004-2006. *Clin Infect Dis* **45**, 1120-1131.

Graziano V., Luo G., Blainey P. C., Perez-Berna A. J., McGrath W. J., Flint S. J., San Martin C., Xie X. S., Mangel W. F. (2013). Regulation of a viral proteinase by a peptide and DNA in one-dimensional space: II. adenovirus proteinase is activated in an unusual one-dimensional biochemical reaction. *J Biol Chem* **288**, 2068-2080.

Greber U. F. (1998). Virus assembly and disassembly: The adenovirus cysteine protease as a trigger factor. *Rev Med Virol* **8**, 213-222.

Greber U. F., Willetts M., Webster P., Helenius A. (1993). Stepwise dismantling of adenovirus 2 during entry into cells. *Cell* **75**, 477-486.

Greber U. F., Webster P., Weber J., Helenius A. (1996). The role of the adenovirus protease on virus entry into cells. *Embo J* **15**, 1766-1777.

Greer A. E., Hearing P., Ketner G. (2011). The adenovirus E4 11k protein binds and relocalizes the cytoplasmic P-body component Ddx6 to aggresomes. *Virology* **417**, 161-168.

Griffin B. D. & Nagy É. (2011). Coding potential and transcript analysis of fowl adenovirus 4: Insight into upstream ORFs as common sequence features in adenoviral transcripts. *J Gen Virol* **92**, 1260-1272.

Groft C. M. & Burley S. K. (2002). Recognition of eIF4G by rotavirus NSP3 reveals a basis for mRNA circularization. *Mol Cell* **9**, 1273-1283.

Grove J. & Marsh M. (2011). The cell biology of receptor-mediated virus entry. *J Cell Biol* **195**, 1071-1082.

Gupta S., Mangel W. F., McGrath W. J., Perek J. L., Lee D. W., Takamoto K., Chance M. R. (2004). DNA binding provides a molecular strap activating the adenovirus proteinase. *Mol Cell Proteomics* **3**, 950-959.

Gyurcsik B., Haruki H., Takahashi T., Mihara H., Nagata K. (2006). Binding modes of the precursor of adenovirus major core protein VII to DNA and template activating factor I: Implication for the mechanism of remodeling of the adenovirus chromatin. *Biochemistry (N Y)* **45**, 303-313.

Haghighat A., Svitkin Y., Novoa I., Kuechler E., Skern T., Sonenberg N. (1996). The eIF4G-eIF4E complex is the target for direct cleavage by the rhinovirus 2A proteinase. *J Virol* **70**, 8444-8450.

Hall T. M. T. (2002). Poly (A) tail synthesis and regulation: Recent structural insights. *Curr Opin Struct Biol* **12**, 82-88.

Hamamori Y., Sartorelli V., Ogryzko V., Puri P. L., Wu H., Wang J. Y., Nakatani Y., Kedes L. (1999). Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. *Cell* **96**, 405-413.

Hammarskjöld M. & Winberg G. (1980). Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. *Cell* **20**, 787-795.

Harada J. N., Shevchenko A., Shevchenko A., Pallas D. C., Berk A. J. (2002). Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery. *J Virol* **76**, 9194-9206.

Harb M., Becker M. M., Vitour D., Baron C. H., Vende P., Brown S. C., Bolte S., Arold S. T., Poncet D. (2008). Nuclear localization of cytoplasmic poly(A)-binding protein upon rotavirus infection involves the interaction of NSP3 with eIF4G and RoXaN. *J Virol* **82**, 11283-11293.

Harpst J. A., Ennever J. F., Russell W. C. (1977). Physical properties of nucleoprotein cores from adenovirus type 5. *Nucleic Acids Res* **4**, 477-490.

Harris M. N., Ozpolat B., Abdi F., Gu S., Legler A., Mawuenyega K. G., Tirado-Gomez M., Lopez-Berestein G., Chen X. (2004). Comparative proteomic analysis of all-trans-retinoic acid treatment reveals systematic posttranscriptional control mechanisms in acute promyelocytic leukemia. *Blood* **104**, 1314-1323.

Hayes B. W., Telling G. C., Myat M. M., Williams J. F., Flint S. J. (1990). The adenovirus L4 100-kilodalton protein is necessary for efficient translation of viral late mRNA species. *J Virol* **64**, 2732-2742.

Hearing P., Samulski R. J., Wishart W. L., Shenk T. (1987). Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J Virol* **61**, 2555-2558.

Hellen C. U. & Sarnow P. (2001). Internal ribosome entry sites in eukaryotic mRNA molecules. *Genes Dev* **15**, 1593-1612.

Henry L. J., Xia D., Wilke M. E., Deisenhofer J., Gerard R. D. (1994). Characterization of the knob domain of the adenovirus type 5 fiber protein expressed in escherichia coli. *J Virol* **68**, 5239-5246.

Hershey J. W. (1991). Translational control in mammalian cells. *Annu Rev Biochem* **60**, 717-755.

Hershey J. W. & Merrick W. C. (2000). 2 the pathway and mechanism of initiation of protein synthesis. *Cold Spring Harbor Monograph Archive* **39**, 33-88.

Higashino F., Pipas J. M., Shenk T. (1998). Adenovirus E4orf6 oncoprotein modulates the function of the p53-related protein, p73. *Proc Natl Acad Sci U S A* **95**, 15683-15687.

Hilleman M. R. & Werner J. H. (1954). Recovery of new agent from patients with acute respiratory illness. *Proc Soc Exp Biol Med* **85**, 183-188.

Hindley C. E., Lawrence F. J., Matthews D. A. (2007). A role for transportin in the nuclear import of adenovirus core proteins and DNA. *Traffic* **8**, 1313-1322.

Ho B., Yu S., Chen J. J., Chang S., Yan B., Hong Q., Singh S., Kao C., Chen H., Su K. (2011). Enterovirus-induced miR-141 contributes to shutoff of host protein translation by targeting the translation initiation factor eIF4E. *Cell Host & Microbe* **9**, 58-69.

Holcik M., Lefebvre C., Yeh C., Chow T., Korneluk R. G. (1999). A new internal-ribosome-entry-site motif potentiates XIAP-mediated cytoprotection. *Nat Cell Biol* **1**, 190-192.

Hong S. S., Szolajska E., Schoehn G., Franqueville L., Myhre S., Lindholm L., Ruigrok R. W., Boulanger P., Chroboczek J. (2005). The 100K-chaperone protein from adenovirus serotype 2 (subgroup C) assists in trimerization and nuclear localization of hexons from subgroups C and B adenoviruses. *J Mol Biol* **352**, 125-138.

Hong J. Y., Lee H. J., Piedra P. A., Choi E. H., Park K. H., Koh Y. Y., Kim W. S. (2001). Lower respiratory tract infections due to adenovirus in hospitalized korean children: Epidemiology, clinical features, and prognosis. *Clin Infect Dis* **32**, 1423-1429.

Horsey E. W., Jakovljevic J., Miles T. D., Harnpicharnchai P., Woolford J. L.,Jr. (2004). Role of the yeast Rrp1 protein in the dynamics of pre-ribosome maturation. *Rna* **10**, 813-827.

Horwitz M. S. (2004). Function of adenovirus E3 proteins and their interactions with immunoregulatory cell proteins. *J Gene Med* **6**, S172-S183.

Horwitz M. S., Scharff M. D., Maizel J. V. (1969). Synthesis and assembly of adenovirus 2: I. polypeptide synthesis, assembly of capsomeres, and morphogenesis of the virion. *Virology* **39**, 682-694.

Horwitz M. S., Valderrama G., Hatcher V., Kern R., DeJong P., Spigland I. (1984). CHARACTERIZATION OF ADENOVIRUS ISOLATES FROM AIDS PATIENTSa. *Ann N Y Acad Sci* **437**, 161-174.

Hosokawa K. & Sung M. T. (1976). Isolation and characterization of an extremely basic protein from adenovirus type 5. *J Virol* **17**, 924-934.

Hsieh J. C., Yoo S. K., Ito J. (1990). An essential arginine residue for initiation of protein-primed DNA replication. *Proc Natl Acad Sci U S A* **87**, 8665-8669.

Hu S. L., Hays W. W., Potts D. E. (1984). Sequence homology between bovine and human adenoviruses. *J Virol* **49**, 604-608.

Huang J., Su W., Jeng K., Chang T., Lai M. M. (2012). Attenuation of 40S ribosomal subunit abundance differentially affects host and HCV translation and suppresses HCV replication. *PLoS Pathog* **8**, e1002766.

Huang J. T. & Schneider R. J. (1991). Adenovirus inhibition of cellular protein synthesis involves inactivation of cap-binding protein. *Cell* **65**, 271-280.

Idamakanti N., Reddy P. S., Babiuk L. A., Tikoo S. K. (1999). Transcription mapping and characterization of 284R and 121R proteins produced from early region 3 of bovine adenovirus type 3. *Virology* **256**, 351-359.

Iftode C. & Flint S. J. (2004). Viral DNA synthesis-dependent titration of a cellular repressor activates transcription of the human adenovirus type 2 IVa2 gene. *Proc Natl Acad Sci U S A* **101**, 17831-17836.

Ilkow C. S., Mancinelli V., Beatch M. D., Hobman T. C. (2008). Rubella virus capsid protein interacts with poly(a)-binding protein and inhibits translation. *J Virol* **82**, 4284-4294.

Imataka H. & Sonenberg N. (1997). Human eukaryotic translation initiation factor 4G (eIF4G) possesses two separate and independent binding sites for eIF4A. *Mol Cell Biol* **17**, 6940-6947.

Imelli N., Ruzsics Z., Puntener D., Gastaldelli M., Greber U. F. (2009). Genetic reconstitution of the human adenovirus type 2 temperature-sensitive 1 mutant defective in endosomal escape. *Virology Journal* **6**, 1.

Jackson R. J., Hellen C. U., Pestova T. V. (2010). The mechanism of eukaryotic translation initiation and principles of its regulation. *Nature Reviews Molecular Cell Biology* **11**, 113-127.

Jang S. K., Krausslich H. G., Nicklin M. J., Duke G. M., Palmenberg A. C., Wimmer E. (1988). A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *J Virol* **62**, 2636-2643.

Joachims M., Van Breugel P. C., Lloyd R. E. (1999). Cleavage of poly(A)-binding protein by enterovirus proteases concurrent with inhibition of translation in vitro. *J Virol* **73**, 718-727.

Kahvejian A., Roy G., Sonenberg N. (2001). The mRNA closed-loop model: The function of PABP and PABP-interacting proteins in mRNA translation. *Cold Spring Harb Symp Quant Biol* **66**, 293-300.

Kapp L. D. & Lorsch J. R. (2004). The molecular mechanics of eukaryotic translation. *Annu Rev Biochem* **73**, 657-704.

Katsafanas G. C. & Moss B. (2007). Colocalization of transcription and translation within cytoplasmic poxvirus factories coordinates viral expression and subjugates host functions. *Cell Host & Microbe* **2**, 221-228.

Keiper B. D., Gan W., Rhoads R. E. (1999). Protein synthesis initiation factor 4G. *Int J Biochem Cell Biol* **31**, 37-41.

Kelsey D. S. (1978). Adenovirus meningoencephalitis. *Pediatrics* **61**, 291-293.

Kerekatte V., Keiper B. D., Badorff C., Cai A., Knowlton K. U., Rhoads R. E. (1999). Cleavage of poly(A)-binding protein by coxsackievirus 2A protease in vitro and in vivo: Another mechanism for host protein synthesis shutoff? *J Virol* **73**, 709-717.

Kim Y. J., Hong J. Y., Lee H. J., Shin S. H., Kim Y. K., Inada T., Hashido M., Piedra P. A. (2003). Genome type analysis of adenovirus types 3 and 7 isolated during successive outbreaks of lower respiratory tract infections in children. *J Clin Microbiol* **41**, 4594-4599.

Kitajewski J., Schneider R. J., Safer B., Munemitsu S. M., Samuel C. E., Thimmappaya B., Shenk T. (1986). Adenovirus VAI RNA antagonizes the antiviral action of interferon by preventing activation of the interferon-induced eIF-2α kinase. *Cell* **45**, 195-200.

Kitchingman G. R. (1985). Sequence of the DNA-binding protein of a human subgroup E adenovirus (type 4): Comparisons with subgroup A (type 12), subgroup B (type 7), and subgroup C (type 5). *Virology* **146**, 90-101.

Klein M., Earley E., Zellat J. (1959). Isolation from cattle of a virus related to human adenovirus. *Proc Soc Exp Biol Med* **102**, 1-4.

Klinge S., Voigts-Hoffmann F., Leibundgut M., Arpagaus S., Ban N. (2011). Crystal structure of the eukaryotic 60S ribosomal subunit in complex with initiation factor 6. *Science* **334**, 941-948.

Komoriya A., Green L. J., Mervic M., Yamada S. S., Yamada K. M., Humphries M. J. (1991). The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine. *J Biol Chem* **266**, 15075-15079.

Kovács E. R. & Benkő M. (2009). Confirmation of a novel siadenovirus species detected in raptors: Partial sequence and phylogenetic analysis. *Virus Res* **140**, 64-70.

Kozak M. (1987). At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* **196**, 947-950.

Kozak M. (1986). Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes. *Cell* **44**, 283-292.

Kozak M. (1978). How do eucaryotic ribosomes select initiation regions in messenger RNA? *Cell* **15**, 1109-1123.

Krilov L. R., Rubin L. G., Frogel M., Gloster E., Ni K., Kaplan M., Lipson S. M. (1990). Disseminated adenovirus infection with hepatic necrosis in patients with human immunodeficiency virus infection and other immunodeficiency states. *Review of Infectious Diseases* **12**, 303-307.

Kulshreshtha V. (2009). Molecular characterization of 33K protein of bovine adenovirus type 3. PhD thesis. University of Saskatchewan, Saskatoon, Sk, Canada.

Kulshreshtha V., Babiuk L. A., Tikoo S. K. (2004). Role of bovine adenovirus-3 33K protein in viral replication. *Virology* **323**, 59-69.

Kulshreshtha V., Ayalew L. E., Islam A., Tikoo S. K. (2014). Conserved arginines of bovine adenovirus-3 33k protein are important for transportin-3 mediated transport and virus replication. *PloS One* **9**, e101216.

Kulshreshtha V. & Tikoo S. K. (2008). Interaction of bovine adenovirus-3 33K protein with other viral proteins. *Virology* **381**, 29-35.

Kuyumcu-Martinez M., Belliot G., Sosnovtsev S. V., Chang K. O., Green K. Y., Lloyd R. E. (2004a). Calicivirus 3C-like proteinase inhibits cellular translation by cleavage of poly(A)-binding protein. *J Virol* **78**, 8172-8182.

Kuyumcu-Martinez N. M., Van Eden M. E., Younan P., Lloyd R. E. (2004b). Cleavage of poly(A)-binding protein by poliovirus 3C protease inhibits host cell translation: A novel mechanism for host translation shutoff. *Mol Cell Biol* **24**, 1779-1790.

Lang S. E. & Hearing P. (2003). The adenovirus E1A oncoprotein recruits the cellular TRRAP/GCN5 histone acetyltransferase complex. *Oncogene* **22**, 2836-2841.

Latorre P., Kolakofsky D., Curran J. (1998). Sendai virus Y proteins are initiated by a ribosomal shunt. *Mol Cell Biol* **18**, 5021-5031.

Lebreton A., Saveanu C., Decourty L., Rain J. C., Jacquier A., Fromont-Racine M. (2006). A functional network involved in the recycling of nucleocytoplasmic pre-60S factors. *J Cell Biol* **173**, 349-360.

Lechner R. L. & Kelly T. J. (1977). The structure of replicating adenovirus 2 DNA molecules. *Cell* **12**, 1007-1020.

Lee J. B., Baxi M. K., Idamakanti N., Reddy P. S., Zakhartchouk A. N., Pyne C., Babiuk L. A., Tikoo S. K. (1998). Genetic organization and DNA sequence of early region 4 of bovine adenovirus type 3. *Virus Genes* **17**, 99-100.

Leen A. M. & Rooney C. M. (2005). Adenovirus as an emerging pathogen in immunocompromised patients. *Br J Haematol* **128**, 135-144.

Lehmberg E., Traina J. A., Chakel J. A., Chang R., Parkman M., McCaman M. T., Murakami P. K., Lahidji V., Nelson J. W., Hancock W. S. (1999). Reversed-phase high-performance liquid chromatographic assay for the adenovirus type 5 proteome. *Journal of Chromatography B: Biomedical Sciences and Applications* **732**, 411-423.

Lehmkuhl H., Smith M., Dierks R. (1975). A bovine adenovirus type 3: Isolation, characterization, and experimental infection in calves. *Arch Virol* **48**, 39-46.

Lehmkuhl H. D. & Hobbs L. (2008). Serologic and hexon phylogenetic analysis of ruminant adenoviruses. *Arch Virol* **153**, 891-897.

Lenaerts L., De Clercq E., Naesens L. (2008). Clinical features and treatment of adenovirus infections. *Rev Med Virol* **18**, 357-374.

Leopold P. L., Kreitzer G., Miyazawa N., Rempel S., Pfister K. K., Rodriguez-Boulan E., Crystal R. G. (2000). Dynein-and microtubule-mediated translocation of adenovirus serotype 5 occurs after endosomal lysis. *Hum Gene Ther* **11**, 151-165.

Li E., Stupack D., Klemke R., Cheresh D. A., Nemerow G. R. (1998). Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. *J Virol* **72**, 2055-2061.

Lichy J. H., Horwitz M. S., Hurwitz J. (1981). Formation of a covalent complex between the 80,000-dalton adenovirus terminal protein and 5'-dCMP in vitro. *Proc Natl Acad Sci U S A* **78**, 2678-2682.

Lin H. L. & Flint S. (2000). Identification of a cellular repressor of transcription of the adenoviral late IVa 2 gene that is unaltered in activity in infected cells. *Virology* **277**, 397-410.

Lin T. A., Kong X., Haystead T. A., Pause A., Belsham G., Sonenberg N., Lawrence J. C.,Jr. (1994). PHAS-I as a link between mitogen-activated protein kinase and translation initiation. *Science* **266**, 653-656.

Lindenbaum J. O., Field J., Hurwitz J. (1986). The adenovirus DNA binding protein and adenovirus DNA polymerase interact to catalyze elongation of primed DNA templates. *J Biol Chem* **261**, 10218-10227.

Liu F. & Green M. R. (1994). Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. .

Liu Y., Vellekamp G., Chen G., Mirza U. A., Wylie D., Twarowska B., Tang J. T., Porter F. W., Wang S., Nagabhushan T. L. (2003). Proteomic study of recombinant adenovirus 5 encoding human p53 by matrix-assisted laser desorption/ionization mass spectrometry in combination with database search. *International Journal of Mass Spectrometry* **226**, 55-69.

Liu G. Q., Babiss L. E., Volkert F. C., Young C. S., Ginsberg H. S. (1985). A thermolabile mutant of adenovirus 5 resulting from a substitution mutation in the protein VIII gene. *J Virol* **53**, 920-925.

Liu H., Jin L., Koh S. B., Atanasov I., Schein S., Wu L., Zhou Z. H. (2010). Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science* **329**, 1038-1043.

López-Lastra M., Ramdohr P., Letelier A., Vallejos M., Vera-Otarola J., Valiente-Echeverría F. (2010). Translation initiation of viral mRNAs. *Rev Med Virol* **20**, 177-195.

Lozinski G. M., Davis G. G., Krous H. F., Billman G. F., Shimizu H., Burns J. C. (1994). Adenovirus myocarditis: Retrospective diagnosis by gene amplification from formalin-fixed, paraffin-embedded tissues. *Hum Pathol* **25**, 831-834.

Lutz P. & Kedinger C. (1996). Properties of the adenovirus IVa2 gene product, an effector of late-phase-dependent activation of the major late promoter. *J Virol* **70**, 1396-1405.

Lutz P., Rosa-Calatrava M., Kedinger C. (1997). The product of the adenovirus intermediate gene IX is a transcriptional activator. *J Virol* **71**, 5102-5109.

Macejak D. G. & Sarnow P. (1991). Internal initiation of translation mediated by the 5′ leader of a cellular mRNA. *Nature* **353**, 90-94.

Mader S., Lee H., Pause A., Sonenberg N. (1995). The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. *Mol Cell Biol* **15**, 4990-4997.

Majumdar R., Bandyopadhyay A., Maitra U. (2003). Mammalian translation initiation factor eIF1 functions with eIF1A and eIF3 in the formation of a stable 40 S preinitiation complex. *J Biol Chem* **278**, 6580-6587.

Makadiya N. (2013). Functional characterization of 100 K protein of bovine adenovirus type 3. PhD thesis. University of Saskatchewan, Saskatoon, Sk, Canada.

Makadiya N., Gaba A., Tikoo S. K. (2015). Cleavage of bovine adenovirus type 3 non-structural 100K protein by protease is required for nuclear localization in infected cells but is not essential for virus replication. *J Gen Virol* **96**, 2749-2763.

Mal A., Poon R. Y., Howe P. H., Toyoshima H., Hunter T., Harter M. L. (1996). Inactivation of p27Kip1 by the viral E1A oncoprotein in TGFbeta-treated cells. *Nature* **380**, 262-265.

Mangel W. F. & San Martín C. (2014). Structure, function and dynamics in adenovirus maturation. *Viruses* **6**, 4536-4570.

Mangel W. F., McGrath W. J., Toledo D. L., Anderson C. W. (1993). Viral DNA and a viral peptide can act as cofactors of adenovirus virion proteinase activity. .

Mangel W. F., Toledo D. L., Ding J., Sweet R. M., McGrath W. J. (1997). Temporal and spatial control of the adenovirus proteinase by both a peptide and the viral DNA. *Trends Biochem Sci* **22**, 393-398.

Mangel W. F., Baniecki M. L., McGrath W. J. (2003). Specific interactions of the adenovirus proteinase with the viral DNA, an 11-amino-acid viral peptide, and the cellular protein actin. *Cell Mol Life Sci* **60**, 2347-2355.

Mangel W. F., Toledo D. L., Brown M. T., Martin J. H., McGrath W. J. (1996). Characterization of three components of human adenovirus proteinase activity in vitro. *J Biol Chem* **271**, 536-543.

Mangus D. A., Evans M. C., Jacobson A. (2003). Poly (A)-binding proteins: Multifunctional scaffolds for the post-transcriptional control of gene expression. *Genome Biol* **4**, 223.

Marcotrigiano J., Gingras A., Sonenberg N., Burley S. K. (1997). Cocrystal structure of the messenger RNA 5′ cap-binding protein (eIF4E) bound to 7-methyl-GDP. *Cell* **89**, 951-961.

Martín B., Sanz R., Aragüés R., Oliva B., Sierra A. (2008). Functional clustering of metastasis proteins describes plastic adaptation resources of breast-cancer cells to new microenvironments†. *Journal of Proteome Research* **7**, 3242-3253.

Martin M., Knipe D., Fields B., Howley P., Griffin D., Lamb R. (2007). Adenoviridae: The viruses and their replication. *Fields' Virology* **2**, 2355-2395.

Martin M. E. & Berk A. J. (1998). Adenovirus E1B 55K represses p53 activation in vitro. *J Virol* **72**, 3146-3154.

Marttila M., Persson D., Gustafsson D., Liszewski M. K., Atkinson J. P., Wadell G., Arnberg N. (2005). CD46 is a cellular receptor for all species B adenoviruses except types 3 and 7. *J Virol* **79**, 14429-14436.

Mathews M. B., Sonenberg N., Hershey J. W. (2007). 1 origins and principles of translational control. *Cold Spring Harbor Monograph Archive* **48**, 1-40.

Matsuo H., Li H., McGuire A. M., Fletcher C. M., Gingras A., Sonenberg N., Wagner G. (1997). Structure of translation factor elF4E bound to m7GDP and interaction with 4E-binding protein. *Nature Structural & Molecular Biology* **4**, 717-724.

Matthews D. & Russell W. (1998a). Adenovirus core protein V is delivered by the invading virus to the nucleus of the infected cell and later in infection is associated with nucleoli. *J Gen Virol* **79**, 1671-1675.

Matthews D. & Russell W. (1998b). Adenovirus core protein V interacts with p32--a protein which is associated with both the mitochondria and the nucleus. *J Gen Virol* **79**, 1677-1685.

Matthews D. A. (2001). Adenovirus protein V induces redistribution of nucleolin and B23 from nucleolus to cytoplasm. *J Virol* **75**, 1031-1038.

Mattson D. (1973a). Adenovirus infection in cattle. *J Am Vet Med Assoc* **163**, 894-896.

Mattson D. E. (1973b). Naturally occurring infection of calves with a bovine adenovirus. *Am J Vet Res* **34**, 623-629.

Mattson D. E., Norman B. B., Dunbar J. R. (1988). Bovine adenovirus type-3 infection in feedlot calves. *Am J Vet Res* **49**, 67-69.

McGrath W. J., Aherne K. S., Mangel W. F. (2002). In the virion, the 11-amino-acid peptide cofactor pVIc is covalently linked to the adenovirus proteinase. *Virology* **296**, 234-240.

McGrath W. J., Baniecki M. L., Li C., McWhirter S. M., Brown M. T., Toledo D. L., Mangel W. F. (2001). Human adenovirus proteinase: DNA binding and stimulation of proteinase activity by DNA. *Biochemistry (N Y)* **40**, 13237-13245.

McSharry B. P., Burgert H. G., Owen D. P., Stanton R. J., Prod'homme V., Sester M., Koebernick K., Groh V., Spies T.& other authors. (2008). Adenovirus E3/19K promotes evasion of NK cell recognition by intracellular sequestration of the NKG2D ligands major histocompatibility complex class I chain-related proteins A and B. *J Virol* **82**, 4585-4594.

Meier O. & Greber U. F. (2004). Adenovirus endocytosis. *J Gene Med* **6**, S152-S163.

Menne T. F., Goyenechea B., Sánchez-Puig N., Wong C. C., Tonkin L. M., Ancliff P. J., Brost R. L., Costanzo M., Boone C., Warren A. J. (2007). The shwachman-bodian-diamond syndrome protein mediates translational activation of ribosomes in yeast. *Nat Genet* **39**, 486-495.

Merrick W. C. (2004). Cap-dependent and cap-independent translation in eukaryotic systems. *Gene* **332**, 1-11.

Michaels M. G., Green M., Wald E. R., Starzl T. E. (1992). Adenovirus infection in pediatric liver transplant recipients. *J Infect Dis* **165**, 170-174.

Miluzio A., Beugnet A., Volta V., Biffo S. (2009). Eukaryotic initiation factor 6 mediates a continuum between 60S ribosome biogenesis and translation. *EMBO Rep* **10**, 459-465.

Mir M. A. & Panganiban A. T. (2008). A protein that replaces the entire cellular eIF4F complex. *Embo J* **27**, 3129-3139.

Mir M. A., Duran W. A., Hjelle B. L., Ye C., Panganiban A. T. (2008). Storage of cellular 5' mRNA caps in P bodies for viral cap-snatching. *Proc Natl Acad Sci U S A* **105**, 19294-19299.

Mittal S. K., Prevec L., Babiuk L. A., Graham F. L. (1992). Sequence analysis of bovine adenovirus type 3 early region 3 and fibre protein genes. *J Gen Virol* **73**, 3295-3300.

Mittal S. K., Tikoo S. K., Van Donkersgoed J., Beskorwayne T., Godson D. L., Babiuk L. A. (1999). Experimental inoculation of heifers with bovine adenovirus type 3. *Can J Vet Res* **63**, 153-156.

Mohankumar V., Dhanushkodi N. R., Raju R. (2011). Sindbis virus replication, is insensitive to rapamycin and torin1, and suppresses akt/mTOR pathway late during infection in HEK cells. *Biochem Biophys Res Commun* **406**, 262-267.

Mohr I. J., Pe'ery T., Mathews M. B. (2007). 20 protein synthesis and translational control during viral infection. *Cold Spring Harbor Monograph Archive* **48**, 545-599.

Morikazu S., Akihisa M., Toshio T., Gihei S. (1987). Phylogenetic relationships between adenoviruses as inferred from nucleotide sequences of inverted terminal repeats. *Gene* **55**, 85-93.

Morris S. J., Scott G. E., Leppard K. N. (2010). Adenovirus late-phase infection is controlled by a novel L4 promoter. *J Virol* **84**, 7096-7104.

Morrison M. D., Onions D. E., Nicolson L. (1997). Complete DNA sequence of canine adenovirus type 1. *J Gen Virol* **78**, 873-878.

Moyer C. L., Besser E. S., Nemerow G. R. (2015). A single maturation cleavage site in adenovirus impacts cell entry and capsid assembly. *J Virol* **90**, 521-532.

Mufson M. A. & Belshe R. B. (1976). A review of adenoviruses in the etiology of acute hemorrhagic cystitis. *J Urol* **115**, 191-194.

Mul Y. M., Verrijzer C. P., van der Vliet P. C. (1990). Transcription factors NFI and NFIII/oct-1 function independently, employing different mechanisms to enhance adenovirus DNA replication. *J Virol* **64**, 5510-5518.

Mysiak M. E., Holthuizen P. E., van der Vliet P. C. (2004). The adenovirus priming protein pTP contributes to the kinetics of initiation of DNA replication. *Nucleic Acids Res* **32**, 3913-3920.

Nagata K., Guggenheimer R. A., Hurwitz J. (1983). Adenovirus DNA replication in vitro: Synthesis of full-length DNA with purified proteins. *Proc Natl Acad Sci U S A* **80**, 4266-4270.

Neill S. D., Hemstrom C., Virtanen A., Nevins J. R. (1990). An adenovirus E4 gene product trans-activates E2 transcription and stimulates stable E2F binding through a direct association with E2F. *Proc Natl Acad Sci U S A* **87**, 2008-2012.

Neumann R., Chroboczek J., Jacrot B. (1988). Determination of the nucleotide sequence for the penton-base gene of human adenovirus type 5. *Gene* **69**, 153-157.

Nevins J. R. & Darnell J. E. (1978). Groups of adenovirus type 2 mRNA's derived from a large primary transcript: Probable nuclear origin and possible common 3' ends. *J Virol* **25**, 811-823.

Nomura H., Sawada Y., Ohtaki S. (1998). Interaction of p27 with E1A and its effect on CDK kinase activity. *Biochem Biophys Res Commun* **248**, 228-234.

Notredame C., Higgins D. G., Heringa J. (2000). T-coffee: A novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**, 205-217.

Numazaki Y., Kumasaka T., Yano N., Yamanaka M., Miyazawa T., Takai S., Ishida N. (1973). Further study on acute hemorrhagic cystitis due to adenovirus type 11. *N Engl J Med* **289**, 344-347.

O'Malley R. P., Mariano T. M., Siekierka J., Mathews M. B. (1986). A mechanism for the control of protein synthesis by adenovirus VA RNA I. *Cell* **44**, 391-400.

Oosterom-Dragon E. A. & Ginsberg H. S. (1981). Characterization of two temperature-sensitive mutants of type 5 adenovirus with mutations in the 100,000-dalton protein gene. *J Virol* **40**, 491-500.

O'Shea C., Klupsch K., Choi S., Bagus B., Soria C., Shen J., McCormick F., Stokoe D. (2005). Adenoviral proteins mimic nutrient/growth signals to activate the mTOR pathway for viral replication. *Embo J* **24**, 1211-1221.

Ostapchuk P. & Hearing P. (2003). Regulation of adenovirus packaging. In *Adenoviruses: Model and Vectors in Virus-Host Interactions*pp. 165-185. Edited by Anonymous : Springer.

Ostapchuk P. & Hearing P. (2008). Adenovirus IVa2 protein binds ATP. *J Virol* **82**, 10290-10294.

Ostapchuk P., Almond M., Hearing P. (2011). Characterization of empty adenovirus particles assembled in the absence of a functional adenovirus IVa2 protein. *J Virol* **85**, 5524-5531.

Ostapchuk P., Anderson M. E., Chandrasekhar S., Hearing P. (2006). The L4 22-kilodalton protein plays a role in packaging of the adenovirus genome. *J Virol* **80**, 6973-6981.

Ostapchuk P., Yang J., Auffarth E., Hearing P. (2005). Functional interaction of the adenovirus IVa2 protein with adenovirus type 5 packaging sequences. *J Virol* **79**, 2831-2838.

Pantelic R. S., Lockett L. J., Rothnagel R., Hankamer B., Both G. W. (2008). Cryoelectron microscopy map of atadenovirus reveals cross-genus structural differences from human adenovirus. *J Virol* **82**, 7346-7356.

Papp Z., Middleton D. M., Mittal S. K., Babiuk L. A., Baca-Estrada M. E. (1997). Mucosal immunization with recombinant adenoviruses: Induction of immunity and protection of cotton rats against respiratory bovine herpesvirus type 1 infection. *J Gen Virol* **78**, 2933-2943.

Pardo-Mateos A. & Young C. (2004). Adenovirus IVa2 protein plays an important role in transcription from the major late promoter in vivo. *Virology* **327**, 50-59.

Parrish S. & Moss B. (2007). Characterization of a second vaccinia virus mRNA-decapping enzyme conserved in poxviruses. *J Virol* **81**, 12973-12978.

Parrish S., Resch W., Moss B. (2007). Vaccinia virus D10 protein has mRNA decapping activity, providing a mechanism for control of host and viral gene expression. *Proc Natl Acad Sci U S A* **104**, 2139-2144.

Patel A. K. & Tikoo S. K. (2006). 293T cells expressing simian virus 40 T antigen are semi-permissive to bovine adenovirus type 3 infection. *J Gen Virol* **87**, 817-821.

Paterson C. P., Ayalew L. E., Tikoo S. K. (2012). Mapping of nuclear import signal and importin α3 binding regions of 52K protein of bovine adenovirus-3. *Virology* **432**, 63-72.

Paterson C. P. (2010). Molecular characterization of 52K protein of bovine adenovirus type 3. PhD thesis. University of Saskatchewan, Saskatoon, Sk, Canada.

Pavia A. T. (2011). Viral infections of the lower respiratory tract: Old viruses, new viruses, and the role of diagnosis. *Clin Infect Dis* **52 Suppl 4**, S284-9.

Pelletier J. & Sonenberg N. (1988). Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature* **334**, 320-325.

Perez-Berna A. J., Mangel W. F., McGrath W. J., Graziano V., Flint J., San Martin C. (2014). Processing of the l1 52/55k protein by the adenovirus protease: A new substrate and new insights into virion maturation. *J Virol* **88**, 1513-1524.

Perez-Berna A. J., Ortega-Esteban A., Menendez-Conejero R., Winkler D. C., Menendez M., Steven A. C., Flint S. J., de Pablo P. J., San Martin C. (2012). The role of capsid maturation on adenovirus priming for sequential uncoating. *J Biol Chem* **287**, 31582-31595.

Perez-Romero P., Tyler R. E., Abend J. R., Dus M., Imperiale M. J. (2005). Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence in vivo and in vitro. *J Virol* **79**, 2366-2374.

Pestova T. V., Lorsch J. R., Hellen C. U. (2007). 4 the mechanism of translation initiation in eukaryotes. *Cold Spring Harbor Monograph Archive* **48**, 87-128.

Pestova T. V., Borukhov S. I., Hellen C. U. (1998). Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. *Nature* **394**, 854-859.

Pestova T. V., Lomakin I. B., Lee J. H., Choi S. K., Dever T. E., Hellen C. U. (2000). The joining of ribosomal subunits in eukaryotes requires eIF5B. *Nature* **403**, 332-335.

Philipson L., Lonberg-Holm K., Pettersson U. (1968). Virus-receptor interaction in an adenovirus system. *J Virol* **2**, 1064-1075.

Phizicky E. M. & Fields S. (1995). Protein-protein interactions: Methods for detection and analysis. *Microbiol Rev* **59**, 94-123.

Piron M., Vende P., Cohen J., Poncet D. (1998). Rotavirus RNA-binding protein NSP3 interacts with eIF4GI and evicts the poly(A) binding protein from eIF4F. *Embo J* **17**, 5811-5821.

Pitcovski J., Mualem M., Rei-Koren Z., Krispel S., Shmueli E., Peretz Y., Gutter B., Gallili G., Michael A., Goldberg D. (1998). The complete DNA sequence and genome organization of the avian adenovirus, hemorrhagic enteritis virus. *Virology* **249**, 307-315.

Plotch S. J., Bouloy M., Ulmanen I., Krug R. M. (1981). A unique cap (m7GpppXm)-dependent influenza virion endonuclease cleaves capped RNAs to generate the primers that initiate viral RNA transcription. *Cell* **23**, 847-858.

Potter R. N., Cantrell J. A., Mallak C. T., Gaydos J. C. (2012). Adenovirus-associated deaths in US military during postvaccination period, 1999-2010. *Emerging Infect Dis* **18**, 507-509.

Preiss T. & W Hentze M. (2003). Starting the protein synthesis machine: Eukaryotic translation initiation. *Bioessays* **25**, 1201-1211.

Prévôt D., Darlix J., Ohlmann T. (2003). Conducting the initiation of protein synthesis: The role of eIF4G. *Biology of the Cell* **95**, 141-156.

Pyronnet S., Imataka H., Gingras A. C., Fukunaga R., Hunter T., Sonenberg N. (1999). Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. *Embo J* **18**, 270-279.

Qin X. & Sarnow P. (2004). Preferential translation of internal ribosome entry site-containing mRNAs during the mitotic cycle in mammalian cells. *J Biol Chem* **279**, 13721-13728.

Querido E., Blanchette P., Yan Q., Kamura T., Morrison M., Boivin D., Kaelin W. G., Conaway R. C., Conaway J. W., Branton P. E. (2001). Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a cullin-containing complex. *Genes Dev* **15**, 3104-3117.

Rancourt C., Keyvani-Amineh H., Sircar S., Labrecque P., Weber J. M. (1995). Proline 137 is critical for adenovirus protease encapsidation and activation but not enzyme activity. *Virology* **209**, 167-173.

Reddy P. S., Chen Y., Idamakanti N., Pyne C., Babiuk L. A., Tikoo S. K. (1999). Characterization of early region 1 and pIX of bovine adenovirus-3. *Virology* **253**, 299-308.

Reddy P. S., Idamakanti N., Zakhartchouk A. N., Baxi M. K., Lee J. B., Pyne C., Babiuk L. A., Tikoo S. K. (1998). Nucleotide sequence, genome organization, and transcription map of bovine adenovirus type 3. *J Virol* **72**, 1394-1402.

Reddy V. S. & Nemerow G. R. (2014). Structures and organization of adenovirus cement proteins provide insights into the role of capsid maturation in virus entry and infection. *Proc Natl Acad Sci U S A* **111**, 11715-11720.

Reich P. R., Forget B. G., Weissman S. M., Rose J. A. (1966). RNA of low molecular weight in KB cells infected with adenovirus type 2. *J Mol Biol* **17**, 428-439.

Rekosh D., Russell W., Bellet A., Robinson A. (1977). Identification of a protein linked to the ends of adenovirus DNA. *Cell* **11**, 283-295.

Remm M., Remm A., Ustav M. (1999). Human papillomavirus type 18 E1 protein is translated from polycistronic mRNA by a discontinuous scanning mechanism. *J Virol* **73**, 3062-3070.

Rogers G. W.,Jr, Richter N. J., Merrick W. C. (1999). Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. *J Biol Chem* **274**, 12236-12244.

Rogers G. W.,Jr, Richter N. J., Lima W. F., Merrick W. C. (2001). Modulation of the helicase activity of eIF4A by eIF4B, eIF4H, and eIF4F. *J Biol Chem* **276**, 30914-30922.

Rohn K., Prusas C., Monreal G., Hess M. (1997). Identification and characterization of penton base and pVIII protein of egg drop syndrome virus. *Virus Res* **47**, 59-65.

Rosa-Calatrava M., Puvion-Dutilleul F., Lutz P., Dreyer D., de The H., Chatton B., Kedinger C. (2003). Adenovirus protein IX sequesters host-cell promyelocytic leukaemia protein and contributes to efficient viral proliferation. *EMBO Rep* **4**, 969-975.

Rosso P., Cortesina G., Sanvito F., Donadini A., Di Benedetto B., Biffo S., Marchisio P. C. (2004). Overexpression of p27BBP in head and neck carcinomas and their lymph node metastases. *Head Neck* **26**, 408-417.

Rowe W. P., Huebner R. J., Gilmore L. K., Parrott R. H., Ward T. G. (1953). Isolation of a cytopathogenic agent from human adenoids undergoing spontaneous degeneration in tissue culture. *Proc Soc Exp Biol Med* **84**, 570-573.

Rozen F., Edery I., Meerovitch K., Dever T. E., Merrick W. C., Sonenberg N. (1990). Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. *Mol Cell Biol* **10**, 1134-1144.

Rubtsova M. P., Sizova D. V., Dmitriev S. E., Ivanov D. S., Prassolov V. S., Shatsky I. N. (2003). Distinctive properties of the 5'-untranslated region of human hsp70 mRNA. *J Biol Chem* **278**, 22350-22356.

Russell W. & Kemp G. (1995). Role of adenovirus structural components in the regulation of adenovirus infection. In *The Molecular Repertoire of Adenoviruses I*pp. 81-98. Edited by Anonymous : Springer.

Russell D. W. & Spremulli L. L. (1979). Purification and characterization of a ribosome dissociation factor (eukaryotic initiation factor 6) from wheat germ. *J Biol Chem* **254**, 8796-8800.

Russell W. C. (2009). Adenoviruses: Update on structure and function. *J Gen Virol* **90**, 1-20.

Ruzindana-Umunyana A., Imbeault L., Weber J. M. (2002). Substrate specificity of adenovirus protease. *Virus Res* **89**, 41-52.

Sachs A. B. & Varani G. (2000). Eukaryotic translation initiation: There are (at least) two sides to every story. *Nature Structural & Molecular Biology* **7**, 356-361.

Sachs A. B., Sarnow P., Hentze M. W. (1997). Starting at the beginning, middle, and end: Translation initiation in eukaryotes. *Cell* **89**, 831-838.

Salaun C., MacDonald A. I., Larralde O., Howard L., Lochtie K., Burgess H. M., Brook M., Malik P., Gray N. K., Graham S. V. (2010). Poly(A)-binding protein 1 partially relocalizes to the nucleus during herpes simplex virus type 1 infection in an ICP27-independent manner and does not inhibit virus replication. *J Virol* **84**, 8539-8548.

San Martín C. (2012). Latest insights on adenovirus structure and assembly. *Viruses* **4**, 847-877.

Sanvito F., Piatti S., Villa A., Bossi M., Lucchini G., Marchisio P. C., Biffo S. (1999). The beta4 integrin interactor p27(BBP/eIF6) is an essential nuclear matrix protein involved in 60S ribosomal subunit assembly. *J Cell Biol* **144**, 823-837.

Sanvito F., Vivoli F., Gambini S., Santambrogio G., Catena M., Viale E., Veglia F., Donadini A., Biffo S., Marchisio P. C. (2000). Expression of a highly conserved protein, p27BBP, during the progression of human colorectal cancer. *Cancer Res* **60**, 510-516.

Schaley J., O'Connor R. J., Taylor L. J., Bar-Sagi D., Hearing P. (2000). Induction of the cellular E2F-1 promoter by the adenovirus E4-6/7 protein. *J Virol* **74**, 2084-2093.

Scheper G. C. & Proud C. G. (2002). Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? *European Journal of Biochemistry* **269**, 5350-5359.

Scheper G. C., Morrice N. A., Kleijn M., Proud C. G. (2001). The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. *Mol Cell Biol* **21**, 743-754.

Schiestl R. H. & Gietz R. D. (1989). High efficiency transformation of intact yeast cells using single stranded nucleic acids as a carrier. *Curr Genet* **16**, 339-346.

Schneider R. J. & Mohr I. (2003). Translation initiation and viral tricks. *Trends Biochem Sci* **28**, 130-136.

Schoenberg D. R. & Maquat L. E. (2009). Re-capping the message. *Trends Biochem Sci* **34**, 435-442.

Sen N., Cao F., Tavis J. E. (2004). Translation of duck hepatitis B virus reverse transcriptase by ribosomal shunting. *J Virol* **78**, 11751-11757.

Senger B., Lafontaine D. L., Graindorge J., Gadal O., Camasses A., Sanni A., Garnier J., Breitenbach M., Hurt E., Fasiolo F. (2001). The nucle (ol) ar Tif6p and Efl1p are required for a late cytoplasmic step of ribosome synthesis. *Mol Cell* **8**, 1363-1373.

Shenk T. (2001). Adenoviridae: The viruses and their replication. In *Fields' Virology*, 4th edn, Edited by B. N. Fields, D. M. Knipe & P. M. Howley. Philadelphia: Lippincott Williams & Wilkins.

Shisler J., Yang C., Walter B., Ware C. F., Gooding L. R. (1997). The adenovirus E3-10.4K/14.5K complex mediates loss of cell surface fas (CD95) and resistance to fas-induced apoptosis. *J Virol* **71**, 8299-8306.

Short J. J., Vasu C., Holterman M. J., Curiel D. T., Pereboev A. (2006). Members of adenovirus species B utilize CD80 and CD86 as cellular attachment receptors. *Virus Res* **122**, 144-153.

Si K. & Maitra U. (1999). The saccharomyces cerevisiae homologue of mammalian translation initiation factor 6 does not function as a translation initiation factor. *Mol Cell Biol* **19**, 1416-1426.

Si K., Chaudhuri J., Chevesich J., Maitra U. (1997). Molecular cloning and functional expression of a human cDNA encoding translation initiation factor 6. *Proc Natl Acad Sci U S A* **94**, 14285-14290.

Sibley S. D., Goldberg T. L., Pedersen J. A. (2011). Detection of known and novel adenoviruses in cattle wastes via broad-spectrum primers. *Appl Environ Microbiol* **77**, 5001-5008.

Smith R. W. & Gray N. K. (2010). Poly(A)-binding protein (PABP): A common viral target. *Biochem J* **426**, 1-12.

Smyth J. A., Benko M., Moffett D. A., Harrach B. (1996). Bovine adenovirus type 10 identified in fatal cases of adenovirus-associated enteric disease in cattle by in situ hybridization. *J Clin Microbiol* **34**, 1270-1274.

Sonenberg N. (1996). mRNA 5 cap binding protein eIF4E and control of cell growth: Translational control (hershey, JWB, mathews, MB, and sonenberg, N., eds) pp. 245–269. .

Sonenberg N. & Hinnebusch A. G. (2009). Regulation of translation initiation in eukaryotes: Mechanisms and biological targets. *Cell* **136**, 731-745.

Song B., Hu S., Darai G., Spindler K. R., Young C. (1996). Conservation of DNA sequence in the predicted major late promoter regions of selected mastadenoviruses. *Virology* **220**, 390-401.

Sprengel J., Schmitz B., Heuss-Neitzel D., Zock C., Doerfler W. (1994). Nucleotide sequence of human adenovirus type 12 DNA: Comparative functional analysis. *J Virol* **68**, 379-389.

Steegenga W. T., Shvarts A., Riteco N., Bos J. L., Jochemsen A. G. (1999). Distinct regulation of p53 and p73 activity by adenovirus E1A, E1B, and E4orf6 proteins. *Mol Cell Biol* **19**, 3885-3894.

Stoneley M., Chappell S. A., Jopling C. L., Dickens M., MacFarlane M., Willis A. E. (2000). C-myc protein synthesis is initiated from the internal ribosome entry segment during apoptosis. *Mol Cell Biol* **20**, 1162-1169.

Strunze S., Engelke M. F., Wang I., Puntener D., Boucke K., Schleich S., Way M., Schoenenberger P., Burckhardt C. J., Greber U. F. (2011). Kinesin-1-mediated capsid

disassembly and disruption of the nuclear pore complex promote virus infection. *Cell Host & Microbe* **10**, 210-223.

Sukarieh R., Sonenberg N., Pelletier J. (2010). Nuclear assortment of eIF4E coincides with shut-off of host protein synthesis upon poliovirus infection. *J Gen Virol* **91**, 1224-1228.

Sussenbach J. S. (1984). The structure of the genome. In *The Adenoviruses*pp. 35-124. Edited by Anonymous : Springer.

Svitkin Y. V., Herdy B., Costa-Mattioli M., Gingras A. C., Raught B., Sonenberg N. (2005). Eukaryotic translation initiation factor 4E availability controls the switch between cap-dependent and internal ribosomal entry site-mediated translation. *Mol Cell Biol* **25**, 10556-10565.

Svitkin Y. V., Pause A., Haghighat A., Pyronnet S., Witherell G., Belsham G. J., Sonenberg N. (2001). The requirement for eukaryotic initiation factor 4A (elF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. *Rna* **7**, 382-394.

Takahashi E., Cohen S. L., Tsai P., Sweeney J. A. (2006). Quantitation of adenovirus type 5 empty capsids. *Anal Biochem* **349**, 208-217.

Tatsis N. & Ertl H. C. (2004). Adenoviruses as vaccine vectors. *Molecular Therapy* **10**, 616-629.

Taylor L. A. & Rose R. E. (1988). A correction in the nucleotide sequence of the Tn903 kanamycin resistance determinant in pUC4K. *Nucleic Acids Res* **16**, 358.

Thanbichler M., Iniesta A. A., Shapiro L. (2007). A comprehensive set of plasmids for vanillate- and xylose-inducible gene expression in caulobacter crescentus. *Nucleic Acids Res* **35**, e137.

Thomas A., Benne R., Voorma H. (1981). Initiation of eukaryotic protein synthesis. *FEBS Lett* **128**, 177-185.

Tihanyi K., Bourbonniere M., Houde A., Rancourt C., Weber J. M. (1993). Isolation and properties of adenovirus type 2 proteinase. *J Biol Chem* **268**, 1780-1785.

Tollefson A. E., Kuppuswamy M., Shashkova E. V., Doronin K., Wold W. S. (2007). Preparation and titration of CsCl-banded adenovirus stocks. *Adenovirus Methods and Protocols: Volume 1: Adenoviruses, Ad Vectors, Quantitation, and Animal Models* , 223-235.

Tollefson A. E., Hermiston T. W., Lichtenstein D. L., Colle C. F., Tripp R. A., Dimitrov T., Toth K., Wells C. E., Doherty P. C., Wold W. S. (1998). Forced degradation of fas inhibits apoptosis in adenovirus-infected cells. *Nature* **392**, 726-730.

Tollefson A. E., Scaria A., Saha S. K., Wold W. S. (1992). The 11,600-MW protein encoded by region E3 of adenovirus is expressed early but is greatly amplified at late stages of infection. *J Virol* **66**, 3633-3642.

Tollefson A. E., Scaria A., Hermiston T. W., Ryerse J. S., Wold L. J., Wold W. S. (1996). The adenovirus death protein (E3-11.6K) is required at very late stages of infection for efficient cell lysis and release of adenovirus from infected cells. *J Virol* **70**, 2296-2306.

Trachsel H., Erni B., Schreier M. H., Staehelin T. (1977). Initiation of mammalian protein synthesis: II. the assembly of the initiation complex with purified initiation factors. *J Mol Biol* **116**, 755-767.

Tribouley C., Lutz P., Staub A., Kedinger C. (1994). The product of the adenovirus intermediate gene IVa2 is a transcriptional activator of the major late promoter. *J Virol* **68**, 4450-4457.

Trotman L. C., Mosberger N., Fornerod M., Stidwill R. P., Greber U. F. (2001). Import of adenovirus DNA involves the nuclear pore complex receptor CAN/Nup214 and histone H1. *Nat Cell Biol* **3**, 1092-1100.

Tyler R. E., Ewing S. G., Imperiale M. J. (2007). Formation of a multiple protein complex on the adenovirus packaging sequence by the IVa2 protein. *J Virol* **81**, 3447-3454.

Ullman A. J. & Hearing P. (2008). Cellular proteins PML and daxx mediate an innate antiviral defense antagonized by the adenovirus E4 ORF3 protein. *J Virol* **82**, 7325-7335.

Unbehaun A., Borukhov S. I., Hellen C. U., Pestova T. V. (2004). Release of initiation factors from 48S complexes during ribosomal subunit joining and the link between establishment of codon-anticodon base-pairing and hydrolysis of eIF2-bound GTP. *Genes Dev* **18**, 3078-3093.

Vagner S., Gensac M. C., Maret A., Bayard F., Amalric F., Prats H., Prats A. C. (1995). Alternative translation of human fibroblast growth factor 2 mRNA occurs by internal entry of ribosomes. *Mol Cell Biol* **15**, 35-44.

Valenzuela D. M., Chaudhuri A., Maitra U. (1982). Eukaryotic ribosomal subunit anti-association activity of calf liver is contained in a single polypeptide chain protein of mr = 25,500 (eukaryotic initiation factor 6). *J Biol Chem* **257**, 7712-7719.

van Breukelen B., Brenkman A. B., Holthuizen P. E., van der Vliet P. C. (2003). Adenovirus type 5 DNA binding protein stimulates binding of DNA polymerase to the replication origin. *J Virol* **77**, 915-922.

van Oostrum J. & Burnett R. M. (1985). Molecular composition of the adenovirus type 2 virion. *J Virol* **56**, 439-448.

Velicer L. F. & Ginsberg H. S. (1970). Synthesis, transport, and morphogenesis of type adenovirus capsid proteins. *J Virol* **5**, 338-352.

Vellekamp G., Porter F. W., Sutjipto S., Cutler C., Bondoc L., Liu Y., Wylie D., Cannon-Carlson S., Tang J. T., Frei A. (2001). Empty capsids in column-purified recombinant adenovirus preparations. *Hum Gene Ther* **12**, 1923-1936.

Vellinga J., Van der Heijdt S., Hoeben R. C. (2005). The adenovirus capsid: Major progress in minor proteins. *J Gen Virol* **86**, 1581-1588.

Vende P., Piron M., Castagne N., Poncet D. (2000). Efficient translation of rotavirus mRNA requires simultaneous interaction of NSP3 with the eukaryotic translation initiation factor eIF4G and the mRNA 3' end. *J Virol* **74**, 7064-7071.

Ventoso I., Blanco R., Perales C., Carrasco L. (2001). HIV-1 protease cleaves eukaryotic initiation factor 4G and inhibits cap-dependent translation. *Proc Natl Acad Sci U S A* **98**, 12966-12971.

Villas-Bôas C., Conceicao T., Ramirez J., Santoro A., Da Poian A., Montero-Lomelí M. (2009). Dengue virus-induced regulation of the host cell translational machinery. *Brazilian Journal of Medical and Biological Research* **42**, 1020-1026.

von der Haar T., Gross J. D., Wagner G., McCarthy J. E. (2004). The mRNA cap-binding protein eIF 4 E in post-transcriptional gene expression. *Nature Structural & Molecular Biology* **11**, 503-511.

Vrati S., Brookes D. E., Boyle D., Both G. W. (1996). Nucleotide sequence of ovine adenovirus tripartite leader sequence and homologues of the IVa2, DNA polymerase and terminal proteins. *Gene* **177**, 35-41.

Vrati S., Boyle D., Kocherhans R., Both G. W. (1995). Sequence of ovine adenovirus homologs for 100K hexon assembly, 33K, pVIII, and fiber genes: Early region E3 is not in the expected location. *Virology* **209**, 400-408.

Wadell G. (2000). Adenoviruses. In *Principles and Practice of Clinical Virology*, 4th edn, pp. 776. Edited by A. J. Zuckerman, J. E. Banatvala & J. R. Pattison. Chichester ; New York: Wiley.

Walls T., Shankar A., Shingadia D. (2003). Adenovirus: An increasingly important pathogen in paediatric bone marrow transplant patients. *The Lancet Infectious Diseases* **3**, 79-86.

Walsh D. & Mohr I. (2011). Viral subversion of the host protein synthesis machinery. *Nature Reviews Microbiology* **9**, 860-875.

Walsh D., Mathews M. B., Mohr I. (2013). Tinkering with translation: Protein synthesis in virus-infected cells. *Cold Spring Harb Perspect Biol* **5**, a012351.

Walsh D., Arias C., Perez C., Halladin D., Escandon M., Ueda T., Watanabe-Fukunaga R., Fukunaga R., Mohr I. (2008). Eukaryotic translation initiation factor 4F architectural alterations accompany translation initiation factor redistribution in poxvirus-infected cells. *Mol Cell Biol* **28**, 2648-2658.

Wang R. & Li K. (2012). Host factors in the replication of positive-strand RNA viruses. *Chang Gung Med J* **35**, 111-124.

Waterhouse A. M., Procter J. B., Martin D. M., Clamp M., Barton G. J. (2009). Jalview version 2--a multiple sequence alignment editor and analysis workbench. *Bioinformatics* **25**, 1189-1191.

Weber J. (1995). Adenovirus endopeptidase and its role in virus infection. In *The Molecular Repertoire of Adenoviruses I*pp. 227-235. Edited by Anonymous : Springer.

Weber J. (1976). Genetic analysis of adenovirus type 2 III. temperature sensitivity of processing viral proteins. *J Virol* **17**, 462-471.

Webster A., Russell S., Talbot P., Russell W. t., Kemp G. (1989). Characterization of the adenovirus proteinase: Substrate specificity. *J Gen Virol* **70**, 3225-3234.

Webster A. & Kemp G. (1993). The active adenovirus protease is the intact L3 23K protein. *J Gen Virol* **74**, 1415-1420.

Webster A., Hay R. T., Kemp G. (1993). The adenovirus protease is activated by a virus-coded disulphide-linked peptide. *Cell* **72**, 97-104.

Webster A., Leith I. R., Hay R. T. (1994). Activation of adenovirus-coded protease and processing of preterminal protein. *J Virol* **68**, 7292-7300.

Webster A., Leith I. R., Nicholson J., Hounsell J., Hay R. T. (1997). Role of preterminal protein processing in adenovirus replication. *J Virol* **71**, 6381-6389.

Weis F., Giudice E., Churcher M., Jin L., Hilcenko C., Wong C. C., Traynor D., Kay R. R., Warren A. J. (2015). Mechanism of eIF6 release from the nascent 60S ribosomal subunit. *Nature Structural & Molecular Biology* .

Weitzman M. D. & Ornelles D. A. (2005). Inactivating intracellular antiviral responses during adenovirus infection. *Oncogene* **24**, 7686-7696.

Wesley A., Pather M., Tait D. (1993). Nosocomial adenovirus infection in a paediatric respiratory unit. *J Hosp Infect* **25**, 183-190.

White E. (2001). Regulation of the cell cycle and apoptosis by the oncogenes of adenovirus. *Oncogene* **20**.

Wickham T. J., Roelvink P. W., Brough D. E., Kovesdi I. (1996). Adenovirus targeted to heparan-containing receptors increases its gene delivery efficiency to multiple cell types. *Nat Biotechnol* **14**, 1570-1573.

Wickham T. J., Mathias P., Cheresh D. A., Nemerow G. R. (1993). Integrins α v β 3 and α v β 5 promote adenovirus internalization but not virus attachment. *Cell* **73**, 309-319.

Wiethoff C. M., Wodrich H., Gerace L., Nemerow G. R. (2005). Adenovirus protein VI mediates membrane disruption following capsid disassembly. *J Virol* **79**, 1992-2000.

Wilhelmi I., Roman E., Sanchez-Fauquier A. (2003). Viruses causing gastroenteritis. *Clinical Microbiology and Infection* **9**, 247-262.

Wilusz C. J., Wormington M., Peltz S. W. (2001). The cap-to-tail guide to mRNA turnover. *Nature Reviews Molecular Cell Biology* **2**, 237-246.

Wodrich H., Cassany A., D'Angelo M. A., Guan T., Nemerow G., Gerace L. (2006). Adenovirus core protein pVII is translocated into the nucleus by multiple import receptor pathways. *J Virol* **80**, 9608-9618.

Wodrich H., Guan T., Cingolani G., Von Seggern D., Nemerow G., Gerace L. (2003). Switch from capsid protein import to adenovirus assembly by cleavage of nuclear transport signals. *Embo J* **22**, 6245-6255.

Wold W. S. M. & Ison M. G. (2013). Adenoviruses. In *Fields Virology*, 6th edn, pp. 1 online resource (2 v. (xx, 2456, I-82. Edited by B. N. Fields, D. M. Knipe, P. M. Howley & I. Ovid Technologies. Philadelphia: Wolters Kluwer Health/Lippincott Williams & Wilkins.

Wood L. C., Ashby M. N., Grunfeld C., Feingold K. R. (1999). Cloning of murine translation initiation factor 6 and functional analysis of the homologous sequence YPR016c in saccharomyces cerevisiae. *J Biol Chem* **274**, 11653-11659.

Wu K., Guimet D., Hearing P. (2013). The adenovirus L4-33K protein regulates both late gene expression patterns and viral DNA packaging. *J Virol* **87**, 6739-6747.

Wu K., Orozco D., Hearing P. (2012). The adenovirus L4-22K protein is multifunctional and is an integral component of crucial aspects of infection. *J Virol* **86**, 10474-10483.

Wu Q. & Tikoo S. K. (2004). Altered tropism of recombinant bovine adenovirus type-3 expressing chimeric fiber. *Virus Res* **99**, 9-15.

Xi Q., Cuesta R., Schneider R. J. (2004). Tethering of eIF4G to adenoviral mRNAs by viral 100k protein drives ribosome shunting. *Genes Dev* **18**, 1997-2009.

Xing L. & Tikoo S. K. (2006). E1A promoter of bovine adenovirus type 3. *J Gen Virol* **87**, 3539-3544.

Yeh-Kai L., Akusjärvi G., Aleström P., Pettersson U., Tremblay M., Weber J. (1983). Genetic identification of a endoproteinase endoded by the adenovirus genome. *J Mol Biol* **167**, 217-222.

Yew P. R., Liu X., Berk A. J. (1994). Adenovirus E1B oncoprotein tethers a transcriptional repression domain to p53. *Genes Dev* **8**, 190-202.

You L. R., Chen C. M., Yeh T. S., Tsai T. Y., Mai R. T., Lin C. H., Lee Y. H. (1999). Hepatitis C virus core protein interacts with cellular putative RNA helicase. *J Virol* **73**, 2841-2853.

Yu Y., Kudchodkar S. B., Alwine J. C. (2005). Effects of simian virus 40 large and small tumor antigens on mammalian target of rapamycin signaling: Small tumor antigen mediates hypophosphorylation of eIF4E-binding protein 1 late in infection. *J Virol* **79**, 6882-6889.

Yueh A. & Schneider R. J. (2000). Translation by ribosome shunting on adenovirus and hsp70 mRNAs facilitated by complementarity to 18S rRNA. *Genes Dev* **14**, 414-421.

Yueh A. & Schneider R. J. (1996). Selective translation initiation by ribosome jumping in adenovirus-infected and heat-shocked cells. *Genes Dev* **10**, 1557-1567.

Zakhartchouk A., Connors W., Van Kessel A., Tikoo S. K. (2004). Bovine adenovirus type 3 containing heterologous protein in the C-terminus of minor capsid protein IX. *Virology* **320**, 291-300.

Zakhartchouk A. N., Reddy P. S., Baxi M., Baca-Estrada M. E., Mehtali M., Babiuk L. A., Tikoo S. K. (1998a). Construction and characterization of E3-deleted bovine adenovirus type 3 expressing full-length and truncated form of bovine herpesvirus type 1 glycoprotein gD. *Virology* **250**, 220-229.

Zakhartchouk A. N., Godson D. L., Babiuk L. A., Tikoo S. K. (2001). 121R protein from the E3 region of bovine adenovirus-3 inhibits cytolysis of mouse cells by human tumor necrosis factor. *Intervirology* **44**, 29-35.

Zakhartchouk A. N., Reddy P. S., Baxi M., Baca-Estrada M. E., Mehtali M., Babiuk L. A., Tikoo S. K. (1998b). Construction and characterization of E3-deleted bovine adenovirus type 3 expressing full-length and truncated form of bovine herpesvirus type 1 glycoprotein gD. *Virology* **250**, 220-229.

Zhang W. & Arcos R. (2005). Interaction of the adenovirus major core protein precursor, pVII, with the viral DNA packaging machinery. *Virology* **334**, 194-202.

Zhang W. & Imperiale M. J. (2003). Requirement of the adenovirus IVa2 protein for virus assembly. *J Virol* **77**, 3586-3594.

Zhang W., Low J. A., Christensen J. B., Imperiale M. J. (2001). Role for the adenovirus IVa2 protein in packaging of viral DNA. *J Virol* **75**, 10446-10454.

Zhao X. (2016). *The Role of Bovine Adenovirus-3 Protein V (pV) in Virus Replication.* PhD thesis. University of Saskatchewan, Saskatoon, Sk, Canada.

Zheng B., Mittal S. K., Graham F. L., Prevec L. (1994). The E1 sequence of bovine adenovirus type 3 and complementation of human adenovirus type 5 E1A function in bovine cells. *Virus Res* **31**, 163-186.

Zhou P., Sun Y., An J. (2009). Interaction between viral proteins and hosts and its disturbance in the cellular responses to ionising radiation. *Int J Radiat Biol* **85**, 587-597.

Zhou Y., Reddy P. S., Babiuk L. A., Tikoo S. K. (2001a). Bovine adenovirus type 3 E1B small protein is essential for growth in bovine fibroblast cells. *Virology* **288**, 264-274.

Zhou Y. & Tikoo S. K. (2001). Analysis of early region 1 of porcine adenovirus type 3. *Virology* **291**, 68-76.

Zhou Y., Pyne C., Tikoo S. K. (2001b). Determination of bovine adenovirus-3 titer based on immunohistochemical detection of DNA binding protein in infected cells. *J Virol Methods* **94**, 147-153.

Zhu Y., Yu Z., Cai H., Gao Y., Dong X., Li Z., Shi H., Meng Q., Lu C., Xue F. (2011). Isolation, identification, and complete genome sequence of a bovine adenovirus type 3 from cattle in china. *Virology Journal* **8**, 1.

Ziff E. B. & Evans R. M. (1978). Coincidence of the promoter and capped 5′ terminus of RNA from the adenovirus 2 major late transcription unit. *Cell* **15**, 1463-1475.

Ziff E. & Fraser N. (1978). Adenovirus type 2 late mRNA's: Structural evidence for 3'-coterminal species. *J Virol* **25**, 897-906.